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Role of VimF in Gingipain Maturation in Porphyromonas gingivalis

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Role of VimF in Gingipain Maturation in *Porphyromonas gingivalis*

by

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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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ABBREVIATIONS

ALPS	Anionic LPS
ATCC	American type culture colletction
Bcp	Bacterioferritin comigratory protein
BHI	Brain Heart Infusion
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFU	Colony forming unit
COPD	Chronic obstructive pulmonary disease
CP	Chronic periodontitis
CR3	Complement receptor 3
CTD	C-terminal domain
CVD	Cardio vascular disease
FimA	Major fimbrial protein A
GTase	Glycosyltransferase
GTs	Glycosyltransferases
HA	Hemagglutinin
HagA	Hemagglutinin protein A
HagB	Hemagglutinin protein B
HRP	Horseraddish peroxidase
IL	Interleukin
IPTG	Isopropyl-beta-D-thiogalactopyranoside

Kb	Kilobases
kDa	Kilodaltons
Kgp	Lysine gingipain/protease
LDS	lithium dodecyl sulfate
LLO	Lipid linked oligosaccharide
LPS	Lipopolysaccharide
Mfa	Minor fimbrial protein
MMP	Matrix Metalloproteinase
MOI	Multiplicity of infection
MOPS	Morpholinepropanesulfonic acid
MS	Mass spectrometry
OD	Optical density
ORF	Open reading frame
PAI2	Plasminogen activator inhibitor 2
PBS	Phosphate buffer saline
PD	Periodontal disease
PDL	Periodontal Ligament
PGE2	Prostaglandin E2
PMN	Polymorpho nuclear cells
PTM	Post translational modification
RA	Rheumatoid arthritis
RecA	Recombinant protein A
RgpA	Arginine gingipain/protease A

RgpB	Arginine gingipain/protease B
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TCEP	tris(2-carboxyethyl)phosphine
TIMP	Tissue inhibitor of metalloproteinase
TLCK	N-a-p-tosyl-L-lysine chloro-methyl ketone
TLR	Toll like receptor
TNF	Tumor necrosis factor
UDP	Uridine di phosphate
VimA	Virulence Modulating Protein A
VimE	Virulence Modulating Protein E
VimF	Virulence Modulating Protein F

BACTERIAL STRAINS

Porphyromonas gingivalis W83	Wild-type, parent strain
Porphyromonas gingivalis FLL95	*vimF* defective mutant of W83
Porphyromonas gingivalis FLL95C’	Complemented *vimF* defect
Porphyromonas gingivalis ATCC 33277	Wild-type, parent strain
Porphyromonas gingivalis FLL476	*vimF* defective mutant of 33277
Porphyromonas gingivalis FLL476C’	Complemented *vimF* defect of 33277
Porphyromonas gingivalis FLL479	*vimF* chimera
Porphyromonas gingivalis FLL1844	*kgp* defective
Porphyromonas gingivalis FLL2024	*rgpA* defective

ABSTRACT OF THE DISSERTATION

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By

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Dr. Hansel M. Fletcher, Chairperson

Gingipain activity in *Porphyromonas gingivalis*, the major etiological agent in adult periodontitis, is post-translationally regulated by unique Vim proteins including VimF, a putative glycosyltransferase. To ascertain the VimF mediated phenotype we first inactivated the *vimF* gene in *P. gingivalis* ATCC 33277 (FLL476), a less virulent fimbriated strain. We observed that the *vimF*-defective mutant (FLL476) showed a phenotype similar to that of the *vimF*-defective mutant (FLL95) in the *P. gingivalis* W83 background. While hemagglutination was not detected and autoaggregationn was reduced, biofilm formation was increased in FLL476. Also, invasive capacity decreased for this mutant. Furthermore, fimbrial structures were missing in FLL476, suggesting the role of VimF in fimbrial processing. We were able to restore the wildtype phenotype by complementing the defect. Secondly, we cloned, expressed and purified the *vimF* gene and demonstrated its ability to glycosylate gingipains. *In vitro* glycosyltransferase activity for rVimF was observed using UDP-galactose and *N*-acetylglucosamine as donor and acceptor substrates, respectively. Further, in the presence of rVimF and UDP-galactose, a 60 kDa protein from the extracellular fraction of FLL95 which was identified my mass spectrometry as Rgp gingipain, immunoreacted with the glycan specific mAb IB5 antibody. Finally, the polyclonal antibody raised against rVimF that did not react

well with native VimF from *P. gingilvalis*, reacted with a 47 kDa protein when the fractions were first deglycosylated before probing with the antibody. Galactose is vital for growing glycan chain leading to maturation/activation of gingipains. Taken together, these results suggest that VimF glycoprotein is a galactosyltransferase that may be specific for gingipain glycosylation.

CHAPTER ONE

INTRODUCTION

Bacteria and Oral Cavity

Bacteria inherently have the ability to colonize various anatomical locations of the human body living either as commensals or pathogens. Pathogenic bacteria are able to overcome host-mediated resistance and establish infection. Oral cavity is colonized with over 700 different species of bacteria, most of which are not cultivated yet [1,2]. Most of the cultivated bacteria belong to the phyla *Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria, Spirochaetes, Synergistetes and Tenericutes* [2]. Two major bacteria mediated infections of the oral cavity are dental carries and periodontal infections. Bacteria that are able to produce copious amounts of acids and dissolve minerals of the teeth cause dental decay. Although transient, loss of minerals is replenished by the flow of saliva, and frequent consumption of sugary food ensures sustained acid production causing this loss. *Streptococcus mutant* and *Lactobacilli* are primary bacteria implicated in dental carries[3]. On the other hand periodontal disease is mediated by organism that are mostly asaccharolytic but able to produce potent proteases which mediate destruction of the periodontium [4]. Periodontal disease begins with periodontal pockets, which offer a favorable niche for colonization by periodontal bacteria leading to forming plaques. While early colonizers of plaques are generally Gram-positive organism and non-pathogenic, late colonizer are predominantly Gram-negative and pathogenic [5]. *Porphyromonas gingivalis* is implicated in adult periodontitis along with two other red

complex organism namely *Tanarella forsythia* and *Treponema denticola*. *Aggregatibacter actinomycetemcommitans* is associated with localized aggressive periodontitis [6]. These bacteria are all Gram negative, anaerobic and asaccharolytic, with virulence factors that include an array of proteolytic enzymes [7]. *P. gingivalis* is considered to be the most important agent causing adult periodontitis [8,9]. Although caries and periodontitis are clearly bacterial diseases, they are not infectious diseases in the classical sense because they result from a complex interaction between the commensal microbiota, host susceptibility and environmental factors such as diet and smoking.

P. gingivalis is now recognized as a keystone microorganism of the oral microflora [10]. This is due to their ability in the oral cavity to both survive as commensal and, at times cause infection using its various pathogenic factors. Recently it was shown that *P. gingivalis*, even at low colonization levels could influence other oral commensals toward an inflammatory environment leading to bone loss. On the other hand, studies using mice model show that *P. gingivalis* by itself is unable to induce disease [11]. Together with the fact that *P. gingivalis* is present as normal flora in healthy individuals [12,13], suggest that pathogenic potential of this organism is not only due to its pathogenic potential but also related to physiological state of the host and association with other oral microbiome [14].

Progression of Periodontal Disease

Periodontal disease develops when the gum gets detached from the teeth due to inflammation mediated by formation of plaques. This invisible, sticky film forms on teeth

when starches and sugars in food interact with bacteria normally found in mouth. Periodical brushing and flossing removes plaques but it re-forms quickly, usually within 24 hours. Plaque that stays on teeth longer than two or three days can harden under the gum-line into tartar (calculus), a white substance that makes plaque more difficult to remove and that acts as a reservoir for bacteria. Initially, plaques may simply irritate and inflame the gingiva, causing gingivitis, the mildest form of periodontal disease. But unchecked ongoing inflammation eventually causes pockets to develop between gums and teeth that fill with plaque, tartar and bacteria. In time, the pockets become deeper and more bacteria accumulate, eventually advancing under gum line. The progressive infection around the teeth leads to loss of tissue and bone ultimately causing exfoliation of the teeth. While gingivitis is prevalent on an average of 3-4 teeth in 50% of adult population in the United States, adult periodontitis, as defined by pocket depth of >4mm seen on an average of 3-4 teeth in 30% of the adult population. Plaque bacteria elaborate various compounds (H2S, NH3, amines, toxins, enzymes, antigens, etc.) that elicit an inflammatory response. This response is usually protective but excessive cytokines produced by an over reacting immune system can cause damage to host tissue leading to loss of periodontal tissue, pocket formation, and loosening and loss of teeth. In addition white blood cells, in response to the plaque, release a family of enzyme called matrix metalloproteinases (MMPs), which are responsible for break down of connective tissue. Other studies using animal models have documented host genetic factors [15] that play a role in causing periodontal disease. In this introduction, I will explore various virulence factors produced by *Porphyromonas gingivalis* and specifically focus on proteases and their role in pathogenesis.

Anatomy of the Periodontium

The main function of the periodontium is to attach the tooth to the bone tissue of the jaws and to maintain the integrity of the surface of the masticatory mucosa of the oral cavity. The periodontium comprises of the following structures – gingiva, periodontal ligaments, root cementum and alveolar bone [See Fig. 1] The gingiva is that part of the masticatory mucosa which covers the alveolar process and surrounds the cervical portion of the teeth. It consists of an epithelial layer and an underlying connective tissue layer called the lamina propria. Two parts of the gingiva are the free gingiva and attached gingiva. Between the free gingiva and the tooth is the gingival sulcus. The gingival epithelium is a stratified squamous epithelium that is an interface between the external environment, which is exposed to bacterial challenges, and the underlying periodontal tissue. The periodontal ligament is an unmineralized connective tissue located between cementum and alveolar bone functioning to sustain the teeth within the jaw and facilitating movement between tooth and bone [16]. Cells of the periodontal ligament are involved in maintenance, repair and regeneration of periodontal tissue. Thus these cells are multipotent composed of heterogeneous cell populations including fibroblasts that can differentiate into either cementum-forming cementoblasts or bone-forming osteoblasts [16]. The cementum is a specialized mineralized tissue covering the root surfaces having many features common to the bone but devoid of blood, lymph and nerve supply. Although it does not undergo physiologic resorption or remodeling like the bone, it is characterized by continual deposition throughout life. Cementum is present in two forms, the acellular extrinsic fiber cementum covering the upper portion of the root and cellular intrinsic fiber cementum covering the lower apical root region. While the acellular

cementum is critical for tooth attachment to the adjacent periodontal ligaments, the cellular cementum containing cementocytes is thought to contribute to the process of repair after damage to the root surface [17]. Alveolar process (alveolar bone) is that portion of maxilla and mandible that supports the roots and disappears if the teeth are lost. It is composed of two parts: the alveolar bone proper that lines the tooth sockets and the supporting bone constituting the rest of bone. The main function of alveolar bone is to distribute and resorb forces generated by mastication and other tooth contacts.

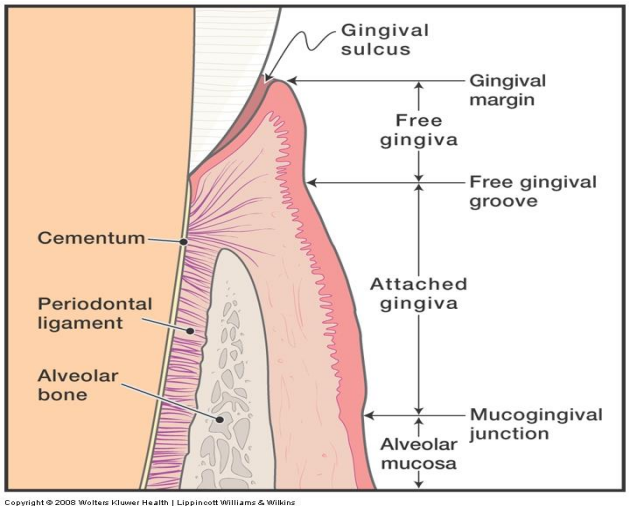

Figure 1. Anatomy of the Periodontium Showing Gingiva, Periodontal Ligaments, Cementum and Alveolar Bone.

Risk Factors of Periodontitis

Many people who carry the periodontal pathogens in the oral cavity do not manifest disease [18]. This important observation highlighted the role of patient susceptibility together with the presence of specific periodontal pathogens in determining the ultimate disease outcome. Added to this are environmental factors such as smoking and stress.

Smoking

It is well accepted that smoking increases the risk for periodontal disease [19,20]. Nicotine, the major content in cigarette has been shown to be involved in osteoclast mediated bone resorption. Nicotine was shown to increase secretion of IL-1β, an osteoclastogenic cytokine, in co-cultures of periodontal ligament (PDL) cells with CD4+T cells [21]. Other studies have shown that nicotine can cause reduction in anti-inflammatory and chemoattractant cytokines, which may be another possible mechanism by which bacteria could survive in the gingival sulcus. Similar observation was made when *P. gingivalis* stimulated dendritic cells showed altered function in the presence of nicotine leading to reduction of proinflammatory cytokines [22,23]. Also, cigarette smoke condensate, the particulate matter of cigarette smoke, which includes nicotine among other chemicals, can increase the collagen-degrading ability of human gingival fibroblasts by altering the production and localization of MMPs and TIMPs. Other studies have associated suppression of B-cell function and immunoglobulin production to cigarette smoking [24]. Finally there have been reports that associate smoking cessation

with improved periodontal health [25]. Taken together cigarette smoking increases the risk for periodontal disease.

Diabetes

Several reports have associated periodontal disease to diabetes [26,27]. Epidemiological data have confirmed that diabetes is a major risk factor for periodontitis, in that susceptibility to periodontitis increased by approximately three fold in people with diabetes [28]. Although mechanisms involved in this link is not clearly understood, it is speculated to involve aspects of immune functioning, neutrophil activity and cytokine biology. Emerging evidence support a two-way relationship between diabetes and periodontitis, with diabetes increasing the risk for periodontitis, and periodontal inflammation negatively affecting glycemic control [29]. Treatment of periodontal disease results in reduction of HbA1C by approximately 0.4% [30]. These studies suggest a comprehensive approach to management of diabetes in periodontal disease patients that includes control of both diabetes and periodontitis.

Stress

Stress is known to affect normal functioning of the immune system and therefore involved in various inflammatory diseases including periodontal disease. Mental stress could also influence life-style and dental hygiene habits. This influence not only decreases frequency and quality of dental hygiene but also leads to increase in the use of tobacco, alcohol consumption and change in food habits leading to a diminution of the general health [31,32]. Given that bacterial invasion is facilitated by poor oral hygiene

and weaker immune response periodontal disease prevails. Moreover, stress is associated with reduced saliva flow and change in composition (IgA) and pH of saliva [32,33]. In gingival cervicular fluid stress has been shown to be a risk factor for gingival inflammation with increase in interleukin-1b (an inflammatory cytokine) levels and the presence of cortisol, which depresses the immune system [34-36]. People with aggressive periodontitis were shown to be more depressed and socially isolated than with control group, highlighting the relationship between aggressive periodontitis and psychosocial stress [37,38]. These studies have clearly shown the two-way relationship exist between periodontal disease and stress.

Genetics

The twin model, considered to be most powerful method to study genetic aspects of any disease, have shown that between 38% and 82% of the population variance for various measurements (attachment loss, pocket depth, gingival index and plaque index) may be attributed to genetic factors [39]. This role of genetics and heritability in chronic periodontitis has been well documented by other different studies [40-42]. However, gene polymorphism in periodontitis has been the primary focus of most of the genetic studies. Various genes have thus been implicated in the susceptibility to chronic periodontitis (CP). For example polymorphism of the IL1 have been considered a risk factor for chronic periodontitis for Caucasian CP patients [43], but the results were not applicable for the worldwide population. Similarly various gene polymorphism including polymorphism of IL1, IL6, IL10, VDR, and CD14 have shown some evidence of association with chronic periodontitis susceptibility in certain population, but, the

conclusion were not generalizable [44]. More research with stricter disease classification, large study cohorts, adjusting for relevant risk factors in CP, and including analysis of multiple genes and polymorphisms are needed to gain clarity of role of genetics in periodontal disease.

Complications of Periodontitis

Various studies have associated periodontal disease with other systemic diseases like cardiovascular disease (CVD), stroke, premature rupture of membrane and respiratory disease. Periodontal disease (PD) is arguable the most common chronic inflammatory disease known to man and inflammation is also the common factor for the systemic disease associated with periodontal disease.

Cardiovascular Disease

Number of research supports moderate relative association between cardiovascular disease (CVD) and periodontal disease assessed clinically and radiographically [45,46]. Although most of these studies used epidemiological basis for their conclusion newer studies have used novel approaches. Systemic antibody levels to periodontal microbes have been evaluated and associated with increased prevalence of coronary heart disease, increased atherosclerosis in the carotid artery and more risk of developing coronary events during10 years of follow-up [47-49]. These studies also correlated well with earlier epidemiological studies [50]. An even more direct approach measured bacteria quantitatively in periodontal plaque for known periodontal pathogen versus non-periodontal pathogen serving as controls [51,52]. The result showed that

carotid atherosclerosis as measured by intima-media thickening was increased with higher levels of the periodontal bacteria [53]. In other interventional studies involving small cohorts it was reported that periodontal treatment lead to improved measures of systemic inflammation or subclinical CVD [54-56]. Two possible mechanisms are proposed linking infection and periodontal disease to CVD. In the direct pathway oral bacteria and their byproducts can gain systemic access via the circulatory system. Toward this several studies have documented bacteremia associated with simple processes like mastication to complex dental procedures like scaling [57,58]. Once gaining systemic access, oral bacteria have the potential to directly influence subclinical mediators of cardiovascular events such as hypercoagulability, atherosclerotic development or both. *P. gingivalis*, the major agent in adult periodontitis, was demonstrated to accelerate atherosclerotic development when administered intravenously and later its DNA was recovered from the aortic tissue in an infected mice [59,60]. Moreover *P. gingivalis* was able to, infect macrophages leading to formation of foam cells and aggregate platelets increasing likelihood of thrombus formation, both leading to atherosclerotic process and ischemic cardiovascular events [61-64]. On the other hand indirect pathways suggests that the presence of periodontal pathogens leads to increased levels of systemic inflammatory markers such as C-reactive protein, antibodies and heat shock proteins, which in turn bring about vascular inflammation and atherosclerosis [65-67].

Preterm/Low Birth Weight Babies

Preterm/low birth weight is the most common adverse outcome of association between pregnancy and periodontal disease. Some studies have used periodontal disease

progression during pregnancy as predictor of more severe adverse pregnancy outcome of very preterm birth, independent of other risk factors [68]. However, inconsistencies have been reported of the efficacy of periodontal treatment on birth outcomes [69]. Preterm/low birth weight births may occur as a result of infection and is mediated indirectly, principally by the translocation of bacterial products, such as endotoxin (lipopolysaccharide), and the action of maternally produced inflammatory mediators. Other studies have noted link a between in utero fetal exposures of oral pathogens to increased neonatal intensive care unit admission and length of stay [70]. Infection with bacteria are said to artificially increase the risk of biomolecules such as prostaglandin E2 (PGE2) and TNF-α, which are normally involved in parturition, leading to premature labor. Other studies have documented increased gingival inflammation, decrease in ratio of peripheral T-helper cells to T-suppressor cells (CD4/CD8), decreased levels of immunoglobulin G to periodontal pathogens during second trimester, ovarian hormone stimulation of prostaglandin (PGE1 and PGE2), and influence of plasminogen activator inhibitor type 2 (PAI2) disrupting the balance of the fibrinolytic system, all these leading to suppressed immune system in the pregnant mother [70]. The rise in sex hormones during pregnancy has direct effects on gingival tissue. While estrogen regulate cell proliferation, differentiation and keratinization, progesterone influences permeability of the microvasculature, alters rate and pattern of collagen production and, finally increases the metabolic breakdown of folate which is crucial for fetal development. Therefore sex hormones may exaggerate the periodontitis mediated effects on the developing fetus in a feedback loop.

Respiratory Infections

Anatomical proximity of the oral cavity to respiratory tract predisposed the respiratory system to infection from oral flora. For example microbes that are normal residents of the oropharynx usually cause community-acquired bacterial pneumonia and other respiratory infections as these bacteria are aspirated into the normally sterile lower respiratory tract [71]. This observation has been accepted as improved oral hygiene resulted in reduction of nosocomial pneumonia in both ventilated an non ventilated patients, linking respiratory infections to poor oral health of inpatients [72,73]. Such cause-and-effect relationship has also been observed between periodontitis and COPD further suggesting close link between poor oral hygiene and periodontal disease to respiratory infections [74,75].

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is characterized by inflammation of the synovial joints that lead to swelling, stiffness, tenderness eventually leading to cartilage damage, bone erosion and joint destruction. Autoimmunity is said to play major role its pathogenicity leading to production of autoantibodies. Two antibodies namely rheumatoid factors, antibody against self-IgG, and anti-citrullinated peptide antibody against common auto antigens expressed within and outside joints are common features of patients with RA. Link between periodontitis and RA have been made [76]. As with inflammatory pathways as mechanism linking periodontal disease to other systemic diseases, so also is inflammatory pathways thought to be the link between RA and periodontal disease [77,78]. Also, presence in the synovial fluid of antibodies to Gram-negative, anaerobic

periodontal pathogens, such as *P. gingivalis*, Prevotella intermedia and Tannerella forsythia suggest their role in RA [79]. Moreover, these pathogens have been isolated from synovial fluid cultures from patients with RA [80,81] and immunity to *P. gingivalis* has been shown to be significantly associated the presence of RA-related autoantibody in individuals at risk for RA [82]. Although there is little to support positive effect of periodontal treatment on RA disease activity [83] there is some evidence to show that periodontal infection and inflammation may hamper the effectiveness of anti-TNF therapy in patients with RA [84,85].

In summary, surrogate markers for chronic periodontitis, such as tooth loss, show relatively consistent but weak associations with multiple systemic conditions. Short-term interventional trials have not supported this cause-and-effect relationship. However, effective treatment of periodontal disease is necessary to achieve oral health goals, as well as to reduce the risk of local chronic inflammation and bacteremia caused by these periodontal pathogens.

Porphyromonas gingivalis **and its Virulence Factors**

P. gingivalis, a major pathogen causing adult periodontitis, is a Gram negative, coccobacilli, which is anaerobic, asaccharolytic, fastidious and nonmotile. It forms black colonies on BHI blood agar supplemented with vitamin K and hemin. This asaccharolytic bacterium derives energy from metabolizing amino acids by producing potent proteases. This is helpful, as they have to survive in deep periodontal pockets where there is no supply of carbohydrates. As a late colonizer in the sub-gingival biofilm it is able to gain access to gingival tissue [86,87]. The black pigmentation is said to be due to their ability

to aggregate heme on its surface [88] as they lack any known siderophores with which bacteria procure iron. We will now consider the virulence factors of *P. gingivalis*.

Fimbriae

The first step in establishing infection is contact with host tissue, in this case gingival epithelial cell. *P. gingivalis* is able to accomplish this with the help of fimbriae. Fimbriae are thin hairy cell protrusions that facilitate adherence to salivary proteins, extracellular matrix, gingival epithelial cells and other bacteria. Initially fimbria helps form biofilm by attaching to other bacteria. Two types of fimbria are reported, namely: Type I (major) fimbriae or FimA, plays a role in colonization and invasion, and Type II (minor) fimbriae Mfa, is said to have higher proinflammatory capacity [9,89]. Invasion occurs when major fimbriae I bind to β1 integrin on the surface of host cells causing rearrangement of actin cytoskeleton leading to internalization [90,91]. Fimbriae-dependent proliferation of gingival epithelial cells ensues following internalization [92]. Interestingly two strains of *P. gingivalis*, W50 and W83 that lack major fimbriae are also able to invade cells [93]. Fimbriae is associated with bone destruction in experimental animal models [94]. Fimbriae are also known to elicit production of pro-inflammatory cytokines (IL-1, IL-6, IL-8, TNF-α) and matrix metalloproteinase MMP-9 [95-98]. TLR2 and TLR4 are the major receptors for the fimbriae mediated activation of host cells. The TLR2 mediated signaling leads to two distinct pathways, one resulting in proinflammatory cytokine production and the other leading to expression of cell adhesion molecules, such as ICAM-1 [99]. On the other hand it is reported that signaling through TLR4 requires additional co-stimulation of CD14 and MD-2 [100]. Also, major fimbriae

were shown to exploit TLR2 signaling in order to interact with complement receptor 3 (CR3) thereby activating the binding capacity of CR3, which allows for internalization of the bacteria in macrophages and reduction of IL-12. All this eventually leads to inhibition of bacterial clearance [101,102].

Lipopolysaccharide

Lipopolysaccharide (LPS) is major component of Gram-negative bacteria that help in pathogenicity. The affinity of LPS to pattern recognition receptors such as TLRs and CD14 is well documented [103,104]. LPS has been shown to stimulate proinflammatory cytokine production, such as IL-1α, IL-1β, IL-6, IL-8, IL-18 and TNFα in monocytes [105-108]. Although *P. gingivalis* LPS is a weak stimulator of cytokines production when compared to LPS of enteropathogenic bacteria, it has the ability to antagonize cytokine-stimulating capacity of other pathogens [106,107,109].

Structurally LPS of *P. gingivalis* is different from other species. While the lipid A of most Gram-negative species is a strong activator of TLR4 response, *P. gingivalis* lipid A is an activator of TLR2 and may even act as antagonist to TLR4 [99,110]. Based on acylation lipid A of *P. gingivalis* is said to be of two varieties namely tetra-acylated and the penta-acylated forms. The penta-acylated lipid A through TLR4 up regulates IL-6 and IL-8 while the tetra-acylated which binds to TLR2 did not induce such significant host response [110-112]. Also TNF-α expression was promptly up regulated by the penta-acylated form and gradually declined afterward. Moreover, when hemin availability was high, a condition reflecting inflammation, penta-acylated lipid A was converted to tetra-acylated lipid A [113]. Therefore by modifying its lipid A structure according to the

microenvironment, *P. gingivalis* may modulate the binding affinity of its LPS to its cognate TLR receptors ultimately modulating inflammatory cytokine production, which may help in its pathogenesis. Additionally a second type of LPS has also been reported from *P. gingivalis* called anionic polysaccharide linked lipid A, A-LPS, shown to involved in cell integrity, serum resistance and associated with Arg-X gingipain [114,115]. Taken together *P. gingivalis* shows ability to manipulate its LPS to suit its survival when inside the human host.

Capsule

As in every pathogenic bacterium another important virulence factor for *P. gingivalis* is its capsule. Based on antibody response to capsular antigen six serotypes of capsule have been reported [116,117]. The capsulated strains are more resistant to phagocytosis by polymorphonuclear cells when compared to non-capsulated strains and exhibited different capacity of adherence to gingival epithelial cells [118,119]. Cytokine production was also different for the different serotypes when exposed to macrophage and dendritic cells [120,121]. The role of capsule in downplaying the innate immune response is supported by a study, which showed that a nonencapsulated knockout strain was more potent inducer of cytokine response when compared to capsulated wild type strain [122]. In murine lesion models, it was shown that capsulated strains were highly invasive producing localized abscess [117]. However, newer studies using in-vitro models could not come to the same conclusion suggesting confounding variables in this study [123]. Further studies are required to conclusively associate capsule with invasive capacity and deregulation of host response.

Gingipains

Gingipains are cysteine protease produced by *P. gingivalis*, which used amino acid metabolism for ATP production, and is found associated to the cell or secreted. Eighty five percent of proteolytic activity of *P. gingivalis* is attributed to their ability to produce gingipains [124]. Based on substrate specificity gingipains are classified into arginine specific (Arg-X) and lysine-specific (Lys-X) gingipains [125,126]. Arg-X gingipain is encoded by two genes *rgpA* and *rgp*B, while Lys-X gingipain is encoded by *kgp*. RgpA and Kgp contain both proteolytic and adhesion domains while RgpB contains only proteolytic domain [125]. Being powerful proteases the gingipains have been shown to cleave several immune molecules, such as CD2, CD4 and CD8, leading to hamper immune response. On the other hand they can stimulate expression of protease-activated receptors in gingival epithelial cells, gingival fibroblast cells and T cells leading to cytokine mediated chronic inflammation seen in periodontitis [127-130]. Gingipains can modulate inflammation by controlling the production of inflammatory and anti-inflammatory cytokines. Some studies have shown that they can stimulate production of inflammatory cytokines like IL-6 and IL-8 thereby enhancing inflammatory response. Others have shown that gingipains can inactivate both anti-inflammatory (IL-4, IL-5) and pro-inflammatory (IL-12, IFN-γ) cytokines.

Gingipain can affect the complement system in many ways. It was shown that gingipains destroy the bactericidal activity of human serum and can interfere with all three pathways (classical, lectin and alternate) of complement system by degrading several complement factors [131]. C5a, an anaphylotoxin, is a crucial component of the complement system and mediates recruitment of PNMs leading to bacterial clearance.

While Arg-X gingipains have been shown to cleave C5 resulting in the release of C5a, Lys-X can inactivate C5a receptor on PNMs there by hindering their recruitment [132,133]. Moreover, it is shown that Arg-X can degrade C3, another important component of complement system [134]. C3b is involved in opsonization of bacterial cells and degrading C3 could render the system incapable of opsonization leading to increased resistance of *P. gingivalis* to bactericidal activity.

Gingipains also mediate other important functions like adherence and vascular permeability leading to its survival in the human host. It has been shown that gingipains can mediate bacterial attachment to host gingival epithelial cells and gingival fibroblasts. Moreover, gingipain can also activate plasma kallikerin and bradykinin or alternatively increase the release of thrombin and prothrombin, leading to increased vascular permeability and PMN influx. Also, their ability to [135-137] degrade fibrinogen would inhibit coagulation, which leads to increased bleeding at the site of infection. This is a strategy profits *P. gingivalis* as it is now able to gain hemin, an important nutritional requirement for survival. Further, gingipains have been shown to disrupt cell-cell and cell-matrix adhesion and induce apoptosis. Recent studies have highlighted the role of gingipain concentration gradient in modulating seemingly opposing functions of inflammatory response. At high concentrations, near the biofilm, gingipains induce apoptosis and attenuate the secretion of proinflammatory mediators, while at low concentrations, deeper into the gingival connective tissue, they stimulate inflammation leading to tissue connective tissue damage and alveolar bone loss, which are hallmarks of periodontitis [138,139].

Glycosylation

The completion of genome sequencing of various organisms has revealed that the amount of genes could not account for the variations observed in the organism. Post translational modifications (PTM) accounting for over 200 covalent modifications that determine the biological, physical and chemical properties of proteins [140] is believed to be involved in this variations observed. Examples of posttranslational modifications include glycosylation, phosphorylation, sulfation, acetylation etc. Glycosylation is a modification mediated by complex enzymatic machinery, by which glycans (sugars) are covalently attached to specific amino acid sites of proteins, besides nucleic acid and lipids. In eukaryotes, protein glycosylation occurs as they pass through the endoplasmic reticulum and the Golgi complex. These glycosylated proteins are transported to cell membrane or secreted from the cell. Glycosylation thus lead to formation of monomeric and multimeric glycan linkages that are essential for cell viability, biochemical communication and normal function. Glycosylation of proteins render important properties where by proteins gain physiological functions like maturation, cell adhesion and trafficking, receptor binding and activation. Dysfunctional glycosylation machinery leads to pathogenesis in infectious diseases and development of chronic diseases like cancer, neurological disorders and severe inflammation [141]. Unlike nucleic acids and proteins, which are synthesized in a linear fashion based on template, glycan synthesis is neither template based nor structurally linear. Branching is therefore common and provides structural diversity.

Glycosylation in Bacteria

It is now well accepted that glycosylation occurs in bacteria and that bacteria may employ hitherto unknown pathways to glycosylate their proteins although classical N and O–glycosylation have also been observed [142,143]. While in N-glycosylation glycans are attached to amide nitrogen of Asn residues, in O-glycosylation the attachment is to hydroxyl oxygen of Ser or Thr. It was found earlier that consensus sequence for N-glycosylation, Asn-X-Ser/Thr, observed in eukaryotes was also found in prokaryotes [144]. However recent studies have shown that bacterial N-glycosylation sequence contain an extended sequence containing Asp or Glu at the -2 position – Asp/Glu-X1-Asn-X2-Ser/Thr, where X1 and X2 represent any amino acid except Pro [145]. Generally N-glycosylation follow stepwise assembly of sugars (mediated by glycosyltransferases) in the cytoplasm, donated by soluble nucleotide-activated sugars, to form an oligosaccharide precursor attached via pyrophosphate to a lipid carrier called lipid linked oligosaccharide (LLO). In bacterial LLO, the lipid is undecaprenol instead of dolichol seen in eukarya and archaea. After assembly of the oligosaccharide, the LLO is flipped from the cytoplasm to face the inner membrane. Finally the oligosaccharide is transferred "en bloc" from the lipid carrier to the acceptor protein by the oligosaccharyltransferase (N-OST) [146]. A common example is the LPS biosynthesis [147]. Interestingly, Neisseria and Pseudomonas pilin glycosylation is said to have oligosaccharyltransferase-mediated *O*-glycosylation pathways instead of the commonly found N-OST pathway [148]. On the other hand *O*-glycosylation in bacteria has been shown to occur either by sequential transfer or by block transfer, similar to N-glycosylation. In *Campylobacter jejuni O*-glycosylation is reported in flagellin formation by sequential transfer of

nucleotide-activated sugars to surface exposed Ser or Thr residues in the flagellin subunit by glycosyltransferases (GTase) [143]. Interestingly in *N. meningitides*, the glycan are assembled on the cytoplasmic side of the inner membrane on a lipid anchor by sequential action of several Pgl proteins and flipped to the periplasm by PglF, where the oligosaccharyltransferase (OTase) transfers the glycans to Ser/Thr residues of pilin subunit PilE [143]. In addition, novels *N*-glycosylation mechanisms have been reported from *Haemophilus influenza*, where the whole *N*-glycosylation process occurs in the cytoplasm instead of the lipid anchor of the inner membrane [149].

Glycosyltransferases

Complex glycans play major roles in biology ranging from development of embryo, function of immune system, microbial pathogenesis and cellular communication. Among the various enzymes that take part in glycobiology of the cell, glycosyltransferases (GTs) are the most important. GTs are enzymes that catalyze the attachment of sugars from activated donors to an aglycone acceptor. Although most GTs are specific for one monosaccharide that they transfer, exceptions are the oligosaccharyltransferase that utilize large lipid linked oligosaccharide as mentioned above. Functionally, GTs comprises one of the most diverse groups of enzyme, found in all domains of life. There are about 87,000 GTs classified into about 91 families according to their amino acid sequence similarities (CAZy database, Carbohydrate-Active enZymes) [150]. In spite of the enormous dissimilarities observed in amino acid sequences between GTs, structurally most of them belong to two superfamilies, namely GT-A and GT-B, each consisting of two domains. Enzymes of GT-A fold have two

dissimilar domains. The N-terminal contains several β sheets, which are each flanked by α-helices (Rossmann folds), which recognizes the sugar-nucleotide donor and, the C-terminal domain comprising largely of mixed β-sheets containing the acceptor-binding site. On the other hand GT-B fold contain two similar Rossmann-like folds. While the N-terminal domain provides the acceptor-binding site, the C-terminal domain is responsible for binding the donor sugar. In both GT-A and GT-B fold, the two domains are connected via a linker region and the active site is located between the two domains [150-152].

Chemistry of Glycosyltransferases

Glycosyltransferases exhibit different modes of action and require different cofactors for its normal function. First, unlike GT-A, GT-B glycosyltransfeases are metal-ion independent. Two common divalent cation cofactors are Mg++ and Mn++. Secondly, most GTs are active at pH range of 5.0 to 7.0. Thirdly, GTs exhibit Michaelis-Menten constants (Kms) for nucleotide sugar substrates in the low micromolar range when assayed invitro. Although Km values for acceptor substrates determined by in vitro studies vary dramatically for various enzymes (low micromolar to low millimolars), in vitro studies are not likely to reflect circumstances found in subcellular compartments. For this reason acceptor Km data of in vitro studies do not exactly represent actual affinities for the natural substrates. Also other factors like site of production of GTs, availability of sugar nucleotide-donors will affect glycosylation occurring in the cell.

Several glycosyltransferases act sequentially to bring about the final glycan structure. Therefore every preceding and ensuing GTs is important for the glycosylation to be complete. The end result is a linear and/or branched polymer composed of

monosaccharide linked to one another. It was because of this precision by which each GTs is able to catalyze its specific function in the presence different donors and acceptors normally found in the cytoplasm, that the one-linkage-one-enzyme paradigm was proposed. Several exceptions to this rule are now evident. Firstly, in members of the α2-8 sialyltransferases, it was found that specific glycosidic linkage might actually be the product of one of several structurally and genetically related enzymes [153]. Secondly, as in α1-3 fucosyltransferase, GTs and synthesize two different glycosidic linkages [154]. Thirdly, as seen in β1-4 galactosyltransferase, the presence of another protein (α-lactalbumin) could change the acceptor specificity from GlcNAc to Glc [155]. Finally, as seen in proteoglycans, some GTs have been shown to catalyze two stepwise glycosyltransferase reaction [141]. Additionally as many mammalian glycosyltransferases have consensus N-glycosylation sequences, as well as serine and threonine residues that can be glycosylated, some studies have shown that these sites are indeed glycosylated. Therefore it is possible that GTs can themselves be glycoproteins. Some have even suggested autoglycosylation of GTs [141].

Glycosyltransferase in *P. gingivalis*

As noted above glycosyltransferases in bacteria play a significant role in normal physiology and specifically with pathogenic ability of bacteria. About twenty glycosyltransferase are present in the genome of *P. gingivalis* W83 and most of them are yet to be characterized. That glycosyltransferase mediated reactions are important for virulence of *P. gingivalis* is highlighted by the fact that virulence factors like LPS, capsular polysaccharide, fimbriae and gingipains are closely associated with

glycosyltransferases, *gtfB*, *PG0106*, *gtfB* and *vimF* [156-159]. In the *gtfB* mutant there was complete loss of surface-associated gingipain proteinases, autoaggregation and biofilm formation due to defective O-LPS and A-LPS biosynthesis [157]. In the PG0106 mutant (which is closely related to *vimF*) involved in capsular biosynthesis it was noted that there was an increase in auto-aggregation increased biofilm formation suggesting surface variations leading to this phenotype [156]. In the *gtfA* mutant, the fimbriae was not mature leading to decrease in auto aggregation and attachment to epithelial cells suggesting role in pathogenicity by regulating adhesion [158]. Finally, similar observations like reduced autoaggregation, increased biofilm formation, reduced gingipain activity that were secreted, observed in the *vimF* mutant suggest their role in pathogenicity [159].

Gingipain Maturation

To avoid untimed activation, gingipain like most proteases, are produced as zymogens awaiting activation at a suitable time. Thus one of the key events in gingipain maturation is the conversion of zymogen to the active enzyme. For RgpB it is shown that three sequential autolytic processing steps at the N- and C-terminus are required for gaining full activity. The first cleavage at Arg -103 was essential for the next two processing at the N-terminal propeptide followed by one at C terminus [160]. Finally after this post-translational modification two forms of RgpB, namely the low molecular weight monomeric secreted RgpBcat and membrane bound RgpB, mt-RgpB, are made. Processing of RgpA and Kgp are more complicated than that of RgpB. RgpA like RgpB have an identical caspase-like catalytic domain (RgpAcat) and a membrane bound

immunoglobulin like domain (mtRgpA) [161]. In addition RgpA also has C-terminal extension containing hemagglutinin-adhesin domain. Kgp has the catalytic domain with selectivity for Lys-Xaa peptide bonds followed by a C-terminal extension similar to RgpA. RgpB lacks the hemagglutinin domains except for a short C-terminal domain, which is similar to RgpA, Kgp and other secretory proteins [162,163]. If gingipain activation requires autoproteolytic mechanisms, questions are raised on factors and mechanisms that may be involved in this crucial process. From our lab we have previously reported that genes of the *vim* locus are involved in this maturation/activation process.

Gingipain Maturation and *vim* Locus

Genes of the *vim* locus include *bcp, recA, vimA, vimE, vimF* and *aroG*. In the wild type *P. gingivalis* W83 mature gingipains were found to be active and mostly cell associated with little secreted into the medium. This was altered in the *vim* defective mutants. In the *vimA* mutant, gingipain activity was reduced by 90% in the exponential phase and 40% in the stationary phase when compared to the wild type W83, and gingipains were mostly found secreted into the medium [164,165]. Also it was shown that VimA could interact with gingipains, RgpA, RgpB and Kgp[166]. Although glycan specific mAb 1B5 antibody did not immunoreact with surface polysaccharide and membrane-associated Rgp gingipain, glycoprotein staining of these fractions was positive suggesting incomplete glycosylation of surface and secreted proteins in the mutant [167]. Similar observation was made in the *vimE* defective mutant also [168]. In addition, the lipopolysaccharide profiles of the *vimA* and *vimE* mutants were truncated in comparison

to that of W83. Further, VimA, in addition to its role in glycosylation and anchorage, is shown to be involved in other virulence functions including oxidative stress resistance, sialylation, acetyl coenzyme A transfer, protein sorting and transport [169]. In the *vimF* defective mutant gingipain activities were reduced more than 90% and, in contrast to the *vimA* mutant, the activity were unaffected by the growth phase. Moreover, nonactive gingipain were found in extracellular protein fraction, with high-molecular-weight proteins that immunoreacted with gingipain-specific antibodies. However, the phosphorylated mannose specific mAb 1B5 antibody did not immunoreact with the nonactive extracellular gingipains [159]. Other genes of the *vim* locus have been shown not to be involved in gingipain maturation. While upstream genes *bcp* and *recA* have been shown to play roles in oxidative stress resistance and DNA repair caused by UV irradiation respectively, downstream gene *aroG* is thought to be involved in survival and pathogenicity through synthesis of aromatic amino acids linked to lipid A biogenesis [169-171]. Taken together it is clear that the *vim* genes are involved in the maturation of gingipains and that they may be involved some way in glycosylation of gingipains and that this step is crucial for anchorage of gingipains to cell surface.

Other Genes Involved in Gingipain Maturation

Apart from the genes of the *vim* locus, others, namely, *porT, sov* and *gppX* have also been shown to be important for maturation of gingipains. While the Sec system is said to transport gingipains across the inner membrane, the Por secretion system (PorSS) has been shown to be involved in transporting various *P. gingivalis* proteins, including gingipains, across the outer membrane, to the exterior. This system includes various Por

proteins and the Sov gene product [172,173]. A common theme for the export of proteins via the PorSS is the consensus CTD which has been found even in gingipain proteins [162,163] although not unique to them [174]. PorSS is said to be a novel secretion system and linked to gliding motility of the phylum Bacteroidetes [173]. Sov is shown to be an outer membrane protein with the C-terminal region found exposed to the extracellular milieu, involved in the modulation of Sov function.

The GppX was discovered when *P. gingivalis* W83 genome was surveyed for homologues of FimS, a two-component sensor histidine kinase. Unlike FimR/FimS, the most studied two component system (TCS) in *P. gingivalis*, GppX has both sensor and response regulator domains in the same protein. Mutants of this gene exhibited characteristic similar to *vim* mutants, in that they formed non-pigmented colonies and had reduced gingipain activity that was mostly extracellular [175]. This protein is found to regulate the post-translational maturation and localization of gingipains [175]. Also, GppX is shown to negatively regulate expression of the *luxS* gene and can therefore be involved in interspecies communication [176]. One of the orthologs of GppX from Tannerella forsythia was shown to upregulate expression of glycosylation-related genes modulating autoaggregation by post-translational modification of T. forsythia cell-surface components [177].

Gingipain Glycosylation

Glycosylation of gingipain is suggested to be an important posttranslational modification contributing to maturation/activation of gingipains adding them to the growing list of bacterial glycoproteins. Biochemical analysis of Rgps have revealed that

the monomeric enzymes are glycoproteins containing between 14% (RgpAcat and RgpB) and 30% (mt-RgpAcat and mt-RgpB) carbohydrate by weight [114]. Monosaccharide composition of RgpA revealed the presence of at least nine different sugars – Arabinose, Rhamanose, Fucose, Manose, Galactose, Glucose, N-acetylgalactosamine, N-acetylglucosamine, and Sialic acid [178]. In a seminal paper Curtis and colleagues demonstrated that gingipains are post translationally modified with carbohydrate additions that are cross-reactive with monoclonal antibody 1B5 [114,179]. This antibody raised against monomeric RgpA reacted with *P. gingivalis* LPS and membrane-associated forms of RgpA and RgpB, and not with heterodimeric HRgpA and soluble forms of RgpB. Chemical deglycosylation of gingipains abolished this immunoreactivity, suggesting the role of glycosylation in maturation of gingipains. Also dephosphorylation of anionic polysaccharide abolished cross reactivity to mAb 1B5. Taken together this phosphorylated branched mannan could be a part of the glycan present on Arg-gingipains that is immuno-reactive to mAb 1B5 [115]. Using this antibody, reports from our lab have linked the Vim proteins to secretion, processing leading to maturation of gingipains. Although glycosylated proteins were detected in the membrane and extracellular fractions from *vimA* and *vimE* defective mutants, they failed to react with mAb 1B5 antibody[167]. Also, in the *vimF* defective mutant, polysaccharide profile appears to be similar to that of the wild type, however 1B5 antibody did not react with the membrane preparations, suggesting carbohydrate modifications [159].

C-terminal Residues of RgpB and Maturation

Various studies have associated the role of C-terminal residue of gingipains in

glycosylation, leading to anchorage to the cell membrane and maturation of gingpains [163,180,181]. Truncation of the last two residues (Valyl-lysine) from C terminus of RgpB, is shown to create an inactive version of the protein that lacks post-translational glycosylation and protein trapped behind the outer membrane [162]. Recently it was shown that residues 692 to 702 of the C-terminal domain of RgpB play an important role in attachment to cell surface [180]. While the role of C-terminal domains in these functions is becoming clearer, there are not enough studies to ascertain the genes involved in glycosylation of gingipains. In one study it was shown that expression of RgpB is itself required for proper glycosylation of monomeric RgpA [178]. Moreover, gene products of *wpbB* and several gene of *porR* locus have been shown to be involved in maturation of gingiapns [179,182]. Although these genes are known to be involved in the synthesis of O-antigen side chains of LPS, isogenic mutants showed characteristic loss of pigmentation, glycan synthesis and glycosylation, with reduced gingipain activity that was mostly soluble, observed by us in the *vim* mutants. Although the *vimA*-defective mutant displayed a similar phenotype as the *porR* mutant, the gingipains in the *vimE* and *vimF* mutants were cell associated and inactive. In addition, unlike *vimA* mutant, late onset of gingipain activity was not observed in *vimE* and *vimF* mutants suggesting other modifications beyond those that facilitate membrane anchorage are needed for activation. *vimF* is annotated as a putative glycosyltransferase and its inactivation resulted in decrease of both lysine-specific and arginine-specific gingipain activity with multiple high molecular weight protein found in the extracellular fraction, which were irrelevant of their growth phase.

Approach of the Study

The work of this thesis focuses on VimF mediated glycosylation of gingipain in *P. gingivalis*, more specifically, this work was started aiming to determine glycosyltransferase function of this protein and to understand the site where this putative glycosyltransferase is able to accomplish its biological function. In the second chapter we focus on the ability of VimF to function as a galactosyltransferase and show its ability to transfer galactose to pro-gingipain species. In addition, this chapter also documents VimF mediated phenotypic changes brought about in two genetic backgrounds namely, W83 and ATCC 33277. Further, questions are raised regarding the glycosylation status of VimF protein. The third chapter will focus on site where this VimF mediated glycosylation takes place. Attempts that were made to localize VimF within the bacteria will be documented. The fourth chapter will be an over all summary of the impact of this study in understanding the molecular aspects of VimF mediated gingipain maturation. Here important outcomes of this study will be brought out in light of biology of this protein and various ways to target this protein for treatment options will be highlighted.

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CHAPTER TWO

IN *PORPHYROMONAS GINGIVALIS* VIMF IS INVOLVED IN GINGIPAIN MATURATION THROUGH THE TRANSFER OF GALACTOSE

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Abstract

Previously, we have reported that gingipain activity in *Porphyromonas gingivalis*, the major causative agent in adult periodontitis, is post-translationally regulated by the unique Vim proteins including VimF, a putative glycosyltransferase. To further characterize VimF, an isogenic mutant defective in this gene in a different *P. gingivalis* genetic background was evaluated. In addition, the recombinant VimF protein was used to further confirm its glycosyltransferase function. The *vimF*-defective mutant (FLL476) in the *P. gingivalis* ATCC 33277 genetic background showed a phenotype similar to that of the *vimF*-defective mutant (FLL95) in the *P. gingivalis* W83 genetic background. While hemagglutination was not detected and autoaggregation was reduced, biofilm formation was increased in FLL476. HeLa cells incubated with *P. gingivalis* FLL95 and FLL476 showed a 45% decrease in their invasive capacity. Antibodies raised against the recombinant VimF protein in immunoreacted only with the deglycosylated native VimF protein from *P. gingivalis*. In vitro glycosyltransferase activity for rVimF was observed using UDP-galactose and N-acetylglucosamine as donor and acceptor substrates, respectively. In the presence of rVimF and UDP-galactose, a 60 kDa protein from the extracellular fraction of FLL95 which was identified by mass spectrometry as Rgp gingipain, immunoreacted with the glycan specific mAb 1B5 antibody. Taken together, these results suggest the VimF glycoprotein is a galactosyltransferase that may be specific for gingipain glycosylation. Moreover, galatose is vital for the growing glycan chain.

Introduction

Porphyromonas gingivalis, a Gram-negative, anaerobic bacterium, is a major etiological agent implicated in adult periodontal disease and is associated with other systemic diseases, including cardiovascular disease [1–5]. This data, taken together, implies a significant impact of this organism on the overall health of humans. The ability of this asaccharolytic bacterium to produce proteases has been shown to contribute significantly toward its pathogenicity [6]. A key element in modulating the pathogenic potential of *P. gingivalis* is the post-translational modification of the major proteases, called gingipains [7]. These consist of arginine-specific (Arg-gingipain [Rgp]) and lysine-specific (Lys-gingipain [Kgp]) cysteine proteases that are both extracellular and cell membrane associated [8]. The maturation pathway of the gingipains including its secretion facilitated by a novel POR secretion system (PorSS) is linked to carbohydrate biosynthesis. This pathway is regulated by several proteins including the PorR, PorT, Sov, Rfa, VimA, VimE, VimF and other components of PorSS [Reviewed in [9–11]]. However, there still remains a gap in our comprehensive understanding of the glycosylation process important in gingipain biogenesis. More specifically, the role of VimF in this process is still unclear.

The *bcp-recA-vimA-vimE-vimF-aroG* operon is essential for the maturation/activation/anchorage of the gingipains and regulation of other virulence factors of *P. gingivalis* [10]. Previously, we have reported that the *vimF* gene can affect the phenotypic expression and distribution of the gingipains in *P. gingivalis* [12]. Using the cloned *vimF* gene, a defective mutant was constructed by allelic exchange in W83. This isogenic mutant designated *P. gingivalis* FLL95, when plated on Brucella blood agar

was non-pigmented and non-hemolytic. In contrast to the parent strain, arginine- and lysine-specific gingipain activities were reduced by approximately 97% and 96%, respectively. These activities were unaffected by the growth phase in contrast to the *vimA*-defective mutant *P. gingivalis* FLL92. Expression of the *rgpA*, *rgpB* and *kgp* gingipain genes were unaffected in *P. gingivalis* FLL95 when compared to the wild-type strain. In non-active gingipain extracellular protein fractions, multiple high molecular weight proteins immunoreacted with gingipain specific antibodies. However, the specific phosphorylated mannan oligosaccharide moiety recognized by the monoclonal antibody 1B5 [13] was absent in gingipains from FLL95. Taken together, these results suggest that the VimF protein which is a putative glycosyltransferase group 1 is involved in the regulation of gingipain biogenesis in *P. gingivalis* through glycosylation.

Glycosyltransferases (GTases) catalyze the transfer of monosaccharide or oligosaccharides primarily from an activated sugar donor (UDP sugars) to various substrates, including carbohydrates, proteins and glycoproteins [14]. Their physiologic significance is further highlighted by the fact that they, along with glycosidases, make up 1 to 2 % of the encoded genes in living organisms [15]. Recently, various reports have associated glycosyltransferases with the biogenesis of several virulence components of *P. gingivalis* like capsule [16], fimbriae [17], lipopolysaccharide [18] and gingipains [12]. The carbohydrate composition of the gingipains which is estimated to be 14% to 30% by weight underscores the importance of glycosylation in their maturation process [13].

The post-translational addition of carbohydrates to the gingipains is highly variable, thus implying a role for multiple factors in this process [11,13]. The attachment of carbohydrates to proteins can be either N- and/or O-linked. The N-linked attachment is

to the amide nitrogen of asparagine facilitated by a consensus amino acid sequence of Asn-X-Ser/Thr (N-X-S/T), where X is any amino acid except proline [19]. The O-glycosidic linkage occurs via glycan attachment to the hydroxyl group of serine (S), or threonine (T) [19]. These attachments of sugar to the amino acid chain and glycans on glycoproteins are both mediated by glycosyltransferses. While several GTases are present in the genome of *P. gingivalis* [20] their specific effect on gingipain maturation is less clear. In this report, we have further characterized the putative glycosyltransferase VimF and demonstrated its ability as a galactosyltransferase involved in glycosylation of the pro-gingipain species in *P. gingivalis*.

Materials and Methods

Bacterial Growth Conditions and Gingipain Assays

All strains of *P. gingivalis* were grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) supplemented with hemin (5 µg/ml), vitamin K (0.5 µg/ml) and cysteine (0.1%). Defibrinated sheep blood (5%) and agar (10%) were used in blood agar plates. Escherichia coli strains were grown in Luria-Bertani (LB) broth. Unless otherwise stated, all cultures were incubated at 37oC. *P. gingivalis* strains were maintained in an anaerobic chamber (Coy Manufacturing, Ann Arbor, MI) in 10% H2, 10% CO2, and 80% N2. Growth rates for *P. gingivalis* and strains were determined spectrophotometrically (optical density at 600 nm [OD600]). Antibiotics were used at the following concentrations: clindamycin, 0.5 µg/ml; erythromycin, 300 µg/ml; and carbenicillin, 50 to 100 µg/ml. Rgp and Kgp activities were determined using the microplate reader (Bio-Rad Laboratories, Hercules, CA) as previously reported [21].

DNA Isolation, Analysis and Cloning of the *vimF* Gene.

Chromosomal DNA was extracted from *P. gingivalis* W83, 33277 and isogenic mutants (Table 2.1) as previously described [22]. Alkaline lysis method was used for plasmid DNA extraction [23]. Electrophoresis of DNA was done using 0.8% agarose gel prepared in TAE buffer as reported elsewhere [12]. The pTrcHis2-TOPO TA expression vector (Invitrogen, Carlsbad, CA) was used for generating the rVimF protein. Briefly, the 1.2-kb *vimF* open reading frame without stop codon was amplified from *P. gingivalis* W83 chromosomal DNA using P1 and P2 oligonucleotide primers (Table 2.2). The amplified fragment was purified using the QIAquick PCR Purification kit (Qiagen, Valencia, CA) then cloned into the pTrcHis2 plasmid vector following the manufacturer's protocol. This recombinant plasmid was then used to transform Top 10 competent cells that were then plated on LB agar containing 50 µg/ml of ampicillin. Recombinant plasmids, named pFLL477 (Table 2.1), isolated from several ampicillin resistant colonies were screened for the correct orientation of the insert using PCR and confirmed by digestion with KpnI and SphI. One randomly chosen ampicillin resistant transformant carrying the recombinant plasmid pFLL477 was chosen for further studies. DNA sequencing was used to confirm the absence of any mutation in the *vimF* ORF.

Purification of rVimF

An overnight culture of the Top 10 cells carrying pFLL477 was used to inoculate two liters of prewarmed LB broth containing 50 µg/ml ampicillin. The culture was then grown at 37^{o}C to the exponential phase (OD600 = 0.6) after which it was induced with 1mM IPTG and further incubated for 5 hours. The cells were harvested by centrifugation

(2,400 g for 20 minutes) and washed twice with 10 mM Tris-HCl at pH 7.4. The cell pellet was suspended in binding buffer (20 mM NaH2PO4, 500 mM NaCl and 40 mM Imidazole) and frozen at -20oC. The cells were thawed and lysed by French pressure cell press with five passes in the presence of Mini EDTA-free protease inhibitor tablets (Roche, Indianapolis, IN) after the first and the last passes. After centrifuging the lysate at 2,400 g for 20 minutes to remove cell debris, the cleared supernatant was further centrifuged at 100,000 g for 1 h. The resultant supernatant was either stored at -80OC and used in a glycosyltransferase assay or, mixed with 1 liter of binding buffer containing 0.5% tween and loaded on to the His-Prep FF 16/10 column (GE Healthcare, Piscataway, NJ) for protein purification. After washing the column twice with two column volumes of wash buffer (same as binding buffer), the bound proteins were eluted with buffer containing 500 mM imidazole, 20 mM NaH2PO4 and 500 mM NaCl. Fractions containing the 50 kDa proteins were pooled, buffer exchanged with 10 mM Tris-HCl (pH 7.4) using 10,000 MW cutoff membrane in an ultrafiltration cell (Amicon Inc., Beverly, MA) and concentrated using a speed vacuum concentrator (Savant Instrument, Inc., Farmingdale, NY).

Production of Rabbit Polyclonal Antibodies Against the rVimF Protein

To avoid the 60 kDa GroEL band that was observed to co-purify with rVimF, the purified rVimF (25 µg/lane) was separated by SDS-PAGE using NuPAGE 4 to 12% Bis-Tris gels and excised for antibody production. A total of approximately 1.2 mg of the rVimF protein was excised from the gels, placed in 1x PBS buffer, and sent to Open Biosystems Inc., Huntsville, AL., for the production of polyclonal rabbit VimF antibodies

by using the manufacturer's standard protocol. Dilutions and efficiency of the antibodies were tested in the laboratory with the purified rVimF. All serum was aliquoted and stored at -80oC.

Preparation of *P. gingivalis* Total Cell and Cell-Free Supernatant Fractions

Total cell lysate and extracellular fractions were collected from *P. gingivalis* W83 and FLL95. Cells were grown to log phase and centrifuged at 10,000g for 30 minutes at 4^{o}C. The proteins from the cell-free supernatant were precipitated with ammonium sulphate (80%). The protein pellet was re-suspended in 10 mM Tris-HCl (pH 7.4) and dialyzed extensively against the same buffer to remove ammonium sulphate. The cell pellet was washed two times with 10 mM Tris-HCl (pH 7.4) and kept at -20^{o}C. The cells were lysed by French Pressure Cell Press (American Instrument Company, Silver Spring, MD) as previously described [12]. Following centrifugation for 10,000 g for 30 minutes to remove cell debris, the supernatant was designated as the total cell lysate.

Purification of Gingipain Protease

The gingipains were purified as previously reported [24] with some modifications. Ammonium sulfate instead of acetone precipitation was used to precipitate the gingipains from the culture supernatant of *P. gingivalis* FLL95 or W83 grown to OD600 of 0.8-1.0. In addition, four columns were used in the following order: Hi Load 16/60 Superdex 200 (GE Healthcare, Piscataway, NJ), DEAE Sepharose FF XK16 anion exchange column (Amersham Bioscience, Piscataway, NJ), Arginine Sepharose column

(GE Healthcare, Piscataway, NJ), followed by the Superdex 200HR 10/30 column (Amersham Bioscience, Piscataway, NJ).

SDS-PAGE and Western Blotting

10% SDS-PAGE gel was used for protein separation of purified rVimF and cell lysates of E.coli and *P. gingivalis* strains. Samples were mixed with approximately 10% NuPAGE reducing agent and 25% 4X LDS buffer and heated for 10 minutes at 72^{o}C. Electrophoresis was done at 130V for 70 minutes and stained with SimplyBlue SafeStain for visualization. Nitrocellulose membrane with pore size 0.45 µm (Schleicher & Schuell, Reviera Beach. FL) was used for blotting using 15 V for 25 min in a Semi-Dry Trans-blot apparatus (Bio-Rad, Hercules, CA). These blots were probed by using either rabbit anti-rVimF antibody (1 in 4000 dilution) or, mouse mAb IB5 (1 in 20 dilution) demonstrated to immunoreact with gingipain-associated sugar moiety [13]. Primary antibody was allowed to react with the membrane for 1 hour and, following 4 washing steps, secondary antibody (HRP conjugated goat anti-rabbit or goat anti-mouse, both in 1 in 4000 dilution) was allowed to react for 30 minutes. Following 2 more washing steps, immunoreactive proteins were detected by the procedure described in the Western Lightning Chemiluminescence Reagent Plus kit (Perkin-Elmer Life Sciences, Boston, MA).

Glycosyltransferase Assay

Calibration Curve

A calibration curve, as previously described [25], was generated to establish the relationship between proton production and change in absorbance of the pH indicator.

The reaction mixture (1 ml final volume) contained 2 mM sodium phosphate buffer (pH 8), 0.01 mM phenol red, 0.1 mM MnCl2, 10 mM N- acetylglucosamine, 100 µl of Top 10 cells expressing pFLL477 lysate and different volumes of HCl (10 mM) was added to get final concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8 mM. The absorbance of the mixture was determined spectrophotometrically (optical density at 557 nm [OD557]). The data points were plotted using GraphPad Prizm 5 software (La Jolla, CA).

To screen for donor and acceptor substrate, 2 mM phosphate buffer (pH 8) containing 0.1 mM phenol red, 0.1 mM MnCl2, 10 mM N-acetylglucosamine (acceptor), 100 µl of crude lysate of Top 10 cells containing pFLL477 and UDP-sugars (galactose or glucose) were added to a final concentration of 2 mM. The absorbance at 557 nm was monitored for each sample at 15s intervals for a total of 60 minutes using the spectrophotometer Beckman DU 650(Beckman Coulter, Brea, CA). Other acceptor substrates used in place of N-acetylglucosamine were – glucose, galactose, lactose, *N*-acetylgalactosamine and mannose. All reactions were carried out at a constant temperature of 37ºC. Enzyme activity was calculated using the GraphPad Prizm 5 enzyme kinetics option by intrapolating the OD557 values from calibration curve. A commercially available bovine β-1, 4 galactosyltransferase (Sigma, St. Louis, MO) was used as positive control and a non-specific Top 10 cell lysate served as negative control. All enzymatic assays were done in triplicate and values averaged.

P. gingivalis Proteins as GTase Acceptor Substrate

Extracellular and whole cell lysates of W83 and FLL95 were used as acceptors in the in-vitro Galactosyl transferase assay [25] in the presence of UDP-galactose (donor).

Briefly, in a total reaction volume of 16 µl, supernatant containing about 7 to 15 µg of *P. gingivalis* extracellular protein (in 10 mM Tris-HCl) was mixed with 5 µg of lysate (containing pFLL477 producing the rVimF protein) and 1 µl of 0.8 mM UDP-galactose. This mixture was incubated at 37^{o}C for 2 hours. Similar reactions omitting rVimF lysate and/or UDP-galactose served as controls. After incubation, the reaction was stopped by adding 4X lithium dodecyl sulphate (LDS) buffer (Invitrogen, Carlsbad, CA), reducing agent and water to make up a final volume of 20 µl. The samples were denatured at 72^{o}C for 10 minutes then separated on 10% SDS-PAGE at 130 V for 70 minutes. Carbohydrate specific modifications were visualized using the glycan specific mAb 1B5 antibody in western blot analysis as described elsewhere [13].

Glycoprotein Staining of rVimF and Gingipains

Purified rVimF along with positive and negative controls provided in the Glycoprotein Staining Kit (Pierce, Rockford, IL) were separated by SDS-PAGE and then transferred to nitrocellulose membrane and stained as per manufacturer's instructions. The membrane was stored in deionized water. Glycoproteins were seen as magenta bands with light pink or colorless background. For glycoprotein staining of gingipains, equivalent amounts of purified proteins (W83 catalytic domain and FLL95 proenzyme) were resolved on a 10% separating gel using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in MOPS (Morpholinepropanesulfonic acid)-SDS running buffer according to manufacturer's instructions. Glycoprotein stain was then performed on the gel using Pierce Glycoprotein Staining Kit as per manufacturer's instructions. An

equivalent gel was stained using SimplyBlue SafeStain (Invitrogen, Carlsbad, CA) for comparison.

Determination of Glycosyl Composition of Proenzyme from FLL95

Protein samples (100 µg) were dried and the monosaccharide composition of the proenzyme from FLL95 was determined by methanolysis and silylation followed by GC-MS analysis of trimethylsilyl (TMS)-methyl glycosides [26], with the addition of a reacetylation step just prior to silylation using 25 µl of methanol, 25 µl of pyridine, and 25 µl of acetic anhydride at room temperature for 15 minutes, in order to detect amino sugars.

Tryptic Digestion and Mass Spectroscopy

SDS-PAGE separated protein bands and spots from 2D gels were excised and subjected to digestion with trypsin. The gel slices were first transferred to low retention epi vials (Fisher, Hampton, NH) and dehydrated using neat acetonitrile for 30 minutes. 20 µl of TCEP (tris(2-carboxyethyl)phosphine) was then added and incubated for 1 hr. at 60^{o}C. In the next step, 40 µl alkylating buffer (200 mM iodoacetamide) was added and incubated at room temperature for one hour. The gel slice was washed in 0.5 ml of neat acetonitrile and re-suspended in another 0.5 ml of neat acetonitrile to dehydrate. Next, digestion buffer containing mass spectroscopy grade trypsin in 50 mM NH4HCO3 was added to attain a 1:20 to 1:50 enzyme/substrate ratio and incubated overnight at 40oC. Digestion was stopped using 10 µl of 10% formic acid. Digested peptides were extracted using standard C18 Zip Tip technology (Millipore, Bedford, MA) according to

manufacturer's protocol. MS analysis of extracted peptide was done as described elsewhere [27].

Inactivation of the *vimF* Gene in *P. gingivalis* ATCC 33277

Fusion PCR, used successfully to inactivate genes in our lab [28,29], was used to inactivate the *vimF* gene in 33277. Briefly, a 1 Kb region upstream of *vimF* was amplified with a 5' overhang that was complementary to a 3' region of *ermF* using primers P3 and P4 (Table 2.2) and, 1Kb downstream of *vimF* was amplified with 3' overhang complementary to 5' end of *ermF* by using primers P5 and P6 (Table 2.2). *ermF* was amplified separately using primer P7 and P8 (Table 2.2). Finally, the purified upstream, downstream and *ermF* fragments were combined in one PCR reaction using primer P3 and P6 to replace *vimF* by ermF by PCR. The fused fragment was purified and electroporated into *P. gingivalis* 33277 cells. The electrotransformed cells were plated on BHI blood agar plate containing 10 µg/ml of erythromycin and incubated for 8 – 10 days. Non-black pigmented colonies on blood agar were screened for the correct gene replacement by PCR and DNA sequencing. One isogenic mutant randomly chosen and designated FLL476 (33277Δ*vimF*) was used for further studies.

Complementation of *vimF* Mutants

PCR mediated gene replacement was used to complement the *vimF* defective mutants. Briefly, using primers P9 and P10 (Table 2.2) the ORF of *vimF* with 500 bp flanking regions of both upstream and downstream was first amplified from *P. gingivalis* W83 and 33277 chromosomal DNA and purified using the QIAEX Gel Extraction Kit

(Qiagen, Valencia, CA). This purified fragment was electroporated into *P. gingivalis* FLL95 or FLL476 cells grown to exponential phase (OD600=0.6). Electroporated cells were incubated for 12 hours in 1 ml of BHI broth then plated on BHI blood agar plates. Plates were then screened after 8 days for black pigmented colonies. These colonies were subsequently checked for the presence of the uninterrupted *vimF* gene. One randomly chosen colony designated *P. gingivalis* FLL95C' or FLL476C' was chosen for further study.

Autoaggregation, Hemagglutination and Biofilm assays

Autoaggregation assays of *P. gingivalis* ATCC 33277 and FLL476 was performed as previously described [30] with slight modification. Briefly, *P. gingivalis* cells in the early to mid-log phase was collected by centrifugation, washed three times with PBS, and then re-suspended in PBS to an OD600 of 1.0. Autoaggregation was monitored by the decrease in OD600 of each suspension over a three hour period at 37^{o}C.

Hemagglutination activity was determined as previously reported [31]. After serially diluting the bacterial suspension in a round bottom 96-well microtiter plates an equal volume of (100 µl) of 1% PBS washed sheep erythrocytes was mixed with each dilution and incubated at 4^{o}C for 3 h. Hemagglutination was visually assessed and the last dilution showing complete hemagglutination was taken as the titer.

Biofilm formation was estimated as previously described [32] with little modification. Briefly, *P. gingivalis* cells grown overnight was washed twice with 1X PBS buffer and re-suspended in BHI-PBS (ratio 1:2) at OD600 of 0.2-0.3. 100 µl of this cell suspension was added to multiple wells of a pre-sterilized 96 well plate, covered and

incubated overnight at 37ºC anaerobically. Next day, free floating cells were aspirated and wells were washed four times with 100 µl of 1X PBS. After drying the plates at 37ºC for 30 minutes, 100 µl of 0.5% (w/v) crystal violet was added to the wells and incubated for 30 minutes at room temperature. After removing the crystal violet solution, the wells were washed four times with 1X PBS and de-stained using 100 µl of 95% ethanol for 30 minutes. The released crystal violet was collected in a cuvette and after adding 500 µl of ddH2O the biofilm formation was measured for each well at OD595.

Electron Microscopic Analysis

Tecnai G2 20 Transmission Electron Microscope was used as previously reported [33], to visualize the surface structure of wild type *P. gingivalis* W83 and ATCC 33277 strains compared with their corresponding respective isogenic mutants FLL95 and FLL476, respectively. Briefly, Formvar-carbon coated grids were prepared; the Formvar support was removed by placing the grids in an atmosphere of solvent vapor. Grids were then placed on a wire mesh in a glass Petri dish, with carbon tetrachloride below the wire mesh. Cultures at OD600 =0.8 was pre-clarified and washed twice with PBS (pH 7.2). The final pellet was dissolved in PBS to get OD600 of 0.7. About 200 µl of the processed sample was loaded into a 500 mesh. The grids were then washed in 0.5% acetic acid then by acetone. The carbon film was broken to free the specimen grid, after which the grid was placed in stain solution - neutral 1% aqueous phosphotungstic acid for 30 seconds. After blotting dry, the grid was examined using the Tecnai G2 20 Transmission Electron Microscope.

Adherence and Antibiotic Protection Assay

The HeLa cells were grown and maintained in the Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), streptomycin (100 IU/ml), and amphotericin B (2.5 mg/ml) (Invitrogen, Carlsbad, CA), at 37^{o}C under 5% CO2 atmosphere. Confluent stock cultures were trypsinized, adjusted to approximately 5 x 103 cells/ml, seeded into 12-well plates (Nunc, Rochester, NY) 1 ml per well and further incubated for 48 h to reach semi-confluency (105 cells per well). Standard antibiotic protection assay was used to quantify invasion [34]. Briefly, an isolated colony harvested from solid agar plate was grown to exponential phase in BHI broth. The cells were centrifuged, washed three times in 1 X PBS, and adjusted to 107 CFU/ml of bacteria (confirmed by colony count) in Dulbecco's modified Eagle's medium. Epithelial cell monolayers were washed three times with PBS, infected with bacteria at a multiplicity of infection (MOI) of 1:100 (105 epithelial cells), and then incubated at 37^{o}C for 90 min under a 5% CO2 atmosphere. Non-adherent bacteria were removed by washing with PBS, while cell surface bound bacteria would be killed with metronidazole (200 µg/ml, 60 min). *P. gingivalis* in general is sensitive to 100 µg/ml metronidazole. After removal of antibiotic, the internalized bacteria were released by osmotic lysis in sterile distilled water with scraping. Lysates were serially diluted, plated (in duplicate) on BHI agar, and incubated for 6 to 10 days. The number of bacterial cells recovered was expressed as percentage of the original inoculum. The number of adherent bacteria was obtained by subtracting the number of intracellular bacteria from the total number of bacteria obtained in the absence of metronidazole.

Results

VimF Defective Mutant Displays a Similar Phenotype in a Different Genetic Background of *P. gingivalis*

Inactivation of the *vimF* gene in *P. gingivalis* W83 resulted in a non-black pigmented isogenic mutant designated *P. gingivalis* FLL95, which showed reduced levels of proteolytic, hemagglutinating and hemolytic activities [12]. To further confirm this phenotype in a different genetic background, a *vimF* deletion mutant in *P. gingivalis* ATCC 33277 was constructed by allelic exchange mutagenesis. Following electroporation and plating on selective medium, several erythromycin-resistant colonies were detected after 5 -7 days of incubation. To compare their phenotypic properties with those of the wild-type 33277 strain, all mutants were plated on Brucella blood agar plates. In contrast to the wild-type, all the isogenic mutants had a non-black pigmented, non-hemolytic phenotype. PCR amplification of chromosomal DNA showed that the *vimF* gene was missing in those isogenic mutants in comparison to the wild-type. One randomly chosen mutant, designated FLL476, was chosen for further characterization. The mutation was further confirmed by DNA sequencing (data not shown). Because of the use of an *ermF* cassette lacking a transcriptional terminator, inactivation of *vimF* did not have any polar effects on the expression of its downstream genes which was confirmed using PCR analysis (data not shown). In FLL476 the growth rate (Fig. 2.1A) and gingipain activity (Fig. 2.1B) were reduced to similar levels as previously observed in *P. gingivalis* FLL95 [12]. Complementation of FLL95 and FLL476 with the wild-type gene, which was confirmed using RT-PCR (data not shown), restored growth rate and gingipain activity to both W83 (Fig. 2.1C and D) and ATCC 33277 (Fig. 2.1A and B) wild type levels.

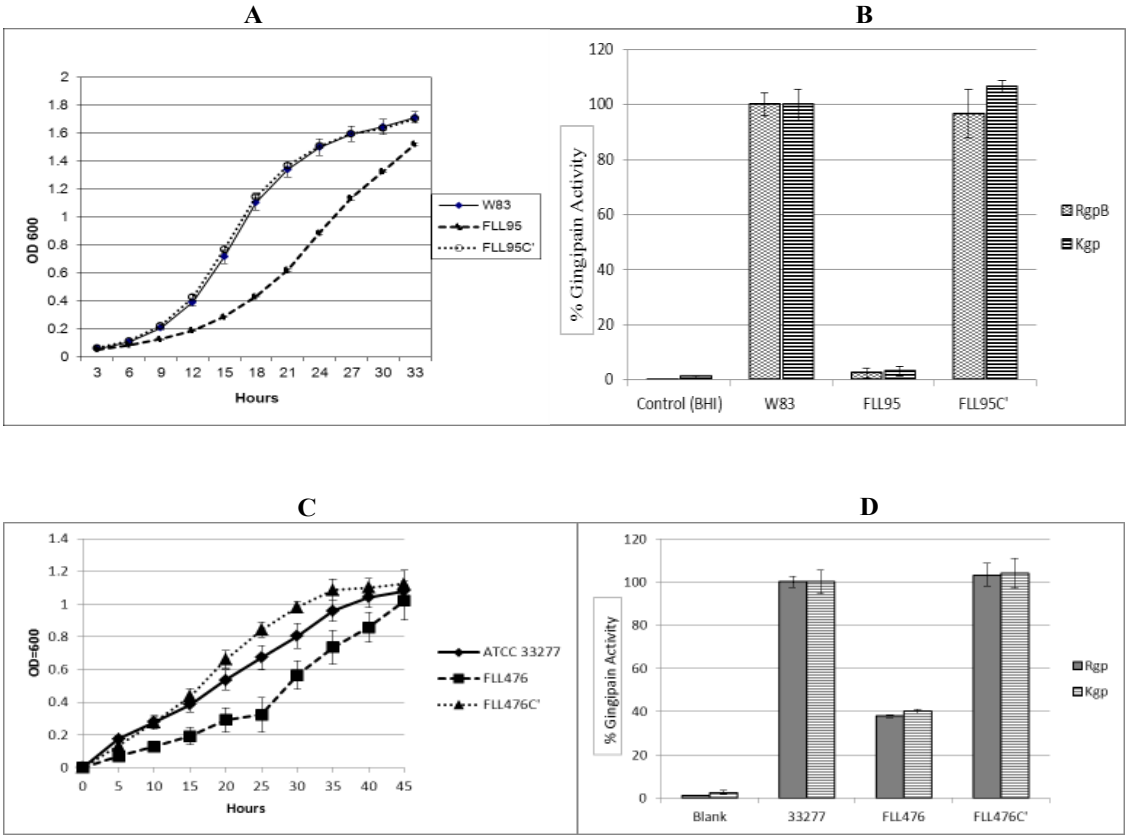


Figure 2.1. Comparison of growth and gingipain activities of wild-type, *vimF* mutant and complemented strains of W83 and ATCC 33277. Growth rate of *P. gingivalis* W83 (**A**) and ATCC 33277 (**C**) were compared with their respective *vimF*-defective isogenic mutants (FLL95 and FLL476) and complemented strains (FLL95C' and FLL476C'). The data shown is an average of three independent replicates. Error bars represent the SD. Gingipain activity of W83 (**B**) and ATCC 33277 (**D**) were compared with respective mutants and complemented strains. The activities were normalized to W83 and ATCC 33277 being 100% and the mutants reported as a percentage thereof. Error bars represent SD.

VimF can Modulate Biofilm Formation, Autoaggregation and Hemagglutination in *P. gingivalis* ATCC 33277

Alteration in *P. gingivalis* cell surface could alter their ability to autoaggregate, hemagglutinate and form biofilm [35–37]. To ascertain the involvement of *vimF* in cell surface modification we evaluated the ability of *vimF* mutant FLL476 to autoaggregate, hemagglutinate and form biofilm. A four-fold increase in biofilm formation was observed in FLL476 when compared to wild-type ATCC 33277 and the complemented strain FLL476C' (Fig. 2.2A). Also, autoaggregation was reduced in FLL476 when compared to ATCC 33277 and FLL476C', however the FLL476C' did not totally regain its autoaggregation ability (Fig. 2.2B). As shown in Fig. 2.2C., hemagglutination was totally abolished in FLL476 when compared to hemagglutination titers of 32 and 64 for 33277 and FLL476C', respectively.

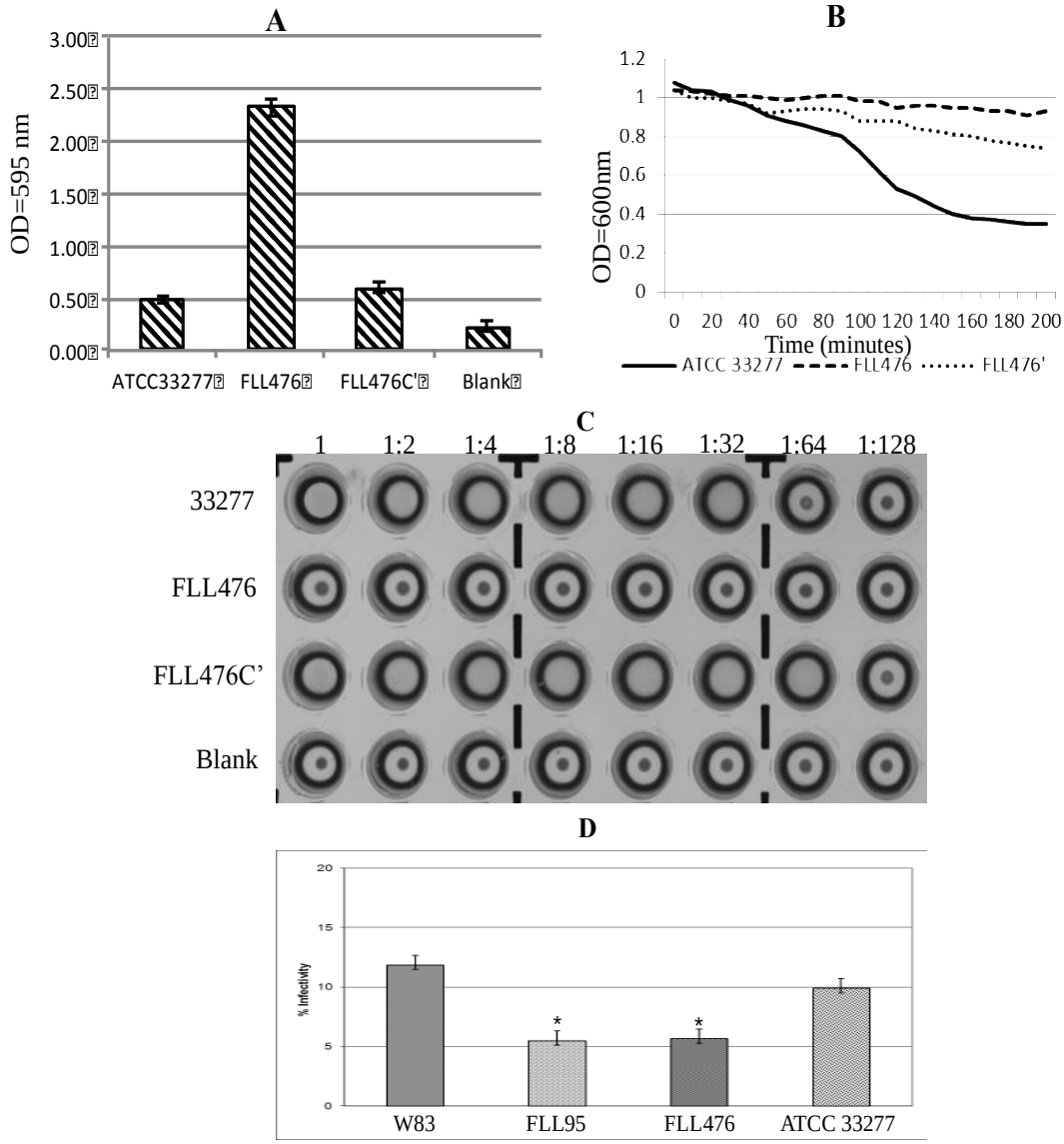


Figure 2.2. Comparison of biofilm formation, autoaggregation, hemagglutination and invasion assay. A. Biofilm formation of ATCC 33277, FLL476 and FLL476C' were compared. Biofilm assay was done by staining adherent cells of overnight cultures grown in microtiter plates with 0.5% (w/v) crystal violet. Blank contained only media. Biofilm forming ability corresponded to OD_{595}. **B**. Autoaggregation of 33277, FLL476 and FLL476C' corresponded to change in OD_{600} monitored for about three hours after cells were washed and suspended in PBS. A representative sample is shown. **C.** Hemagglutination activities of ATCC 33277, FLL476 and FLL476C' were assessed by serially diluting cells in PBS and incubating with sheep RBCs for 3 h at 4ºC. Dilutions are listed above and last dilution showing matt formation was taken as the titre. The blank contained only media. **D.** Antibiotic Protection Assay was used to quantify invasion. *P. gingivalis* cells that were able to invade HeLa cell monolayers were released by lysis and cultured on BA plates. Infectivity was taken as the percentage of cells recovered. (* = $p<0.05$)

VimF can Modulate the Invasive Capacity of *P. gingivalis*

HeLa cells incubated with *P. gingivalis* FLL95 and FLL476 showed a decrease in invasion of approximately 45% compared to the wild-type (Fig. 2.2D). *P. gingivalis* FLL95 complemented with the wild-type gene restored its invasive capacity similar to the parent strain (data not shown).

The Cell Surface is Altered in the *vimF*-defective Isogenic Mutant

Electron microscopy was used to evaluate the cell surface ultra-structure of the wild-type compared to the *vimF*-defective mutants. The wild-type W83 parent strain revealed well defined outer membrane with outer membrane vesicles (Fig. 2.3) that were missing in the isogenic mutant FLL95. The outer membrane and membrane vesicles was restored in the complemented strain, FLL95C'. Electron micrographs of ATCC 33277 and its isogenic mutant FLL476 revealed a modified cell surface that was devoid of fimbria in the FLL476 mutant. The wild-type phenotype was largely restored in the complemented strains.

Cloning, Expression and Purification of rVimF

The *vimF* ORF was cloned into a His-tag containing expression vector. The expected 50 kDa rVimF (47 kDa VimF and 3 kDa for the 6X Histidine tag) was not observed to be secreted but was shown to co-purify with GroEL (60 kDa) only in cell lysates. The purified rVimF protein showed a single band near the 50 kDa region (Fig. 2.4A). However western blot using anti-rVimF antibody showed reactive bands also at 100 kDa and 200 kDa regions (Fig. 2.4B). These two bands corresponding to the multimeric forms of rVimF were confirmed using anti-rVimF antibodies and mass

spectroscopy (data not shown). 2D gel electrophoresis of the purified rVimF showed isoforms near the 50 and 100 kDa regions which were identified as VimF by mass spectroscopy (data not shown). Since glycosylation of proteins is a common cause for the isoforms observed in 2D gels, we used a glycoprotein stain to test whether rVimF was glycosylated. When compared with positive and negative controls for glycoprotein staining, rVimF did not take up the glycoprotein stain (Fig. 2.4D and C).

The Native VimF May be Post-Translationally Modified

Polyclonal antibodies raised against the gel purified rVimF immunoreacted with multiple proteins bands representing the rVimF multimeric forms (Fig. 2.4B). Immune serum did not immunoreact with any other protein (Fig. 2.4B). Similar immunoreactive bands observed with the immune serum were missing using the pre-immune serum in Western blot analysis (data not shown). To determine if the antibodies against the recombinant VimF protein can recognize the native protein, cell lysates from *P. gingivalis* were separated by SDS-PAGE and probed with the anti-rVimF antibodies. As shown in Fig. 2.4E, immunoreactive bands from the *P. gingivalis* cell lysates were missing using the immune serum. However, immunoreactive bands with sizes of 47, 60 and 80 kDa were observed when cell lysate proteins from W83 were first deglycosylated then separated by SDS-PAGE and probed with the anti-rVimF antibodies (Fig. 2.4E). The 47 kDa band corresponds to native VimF. Taken together, these results suggest that differences may exist between the glycosylation of the native and recombinant VimF protein. It is noteworthy that the rVimF was negative for glycoprotein stain (Fig. 2.4D).

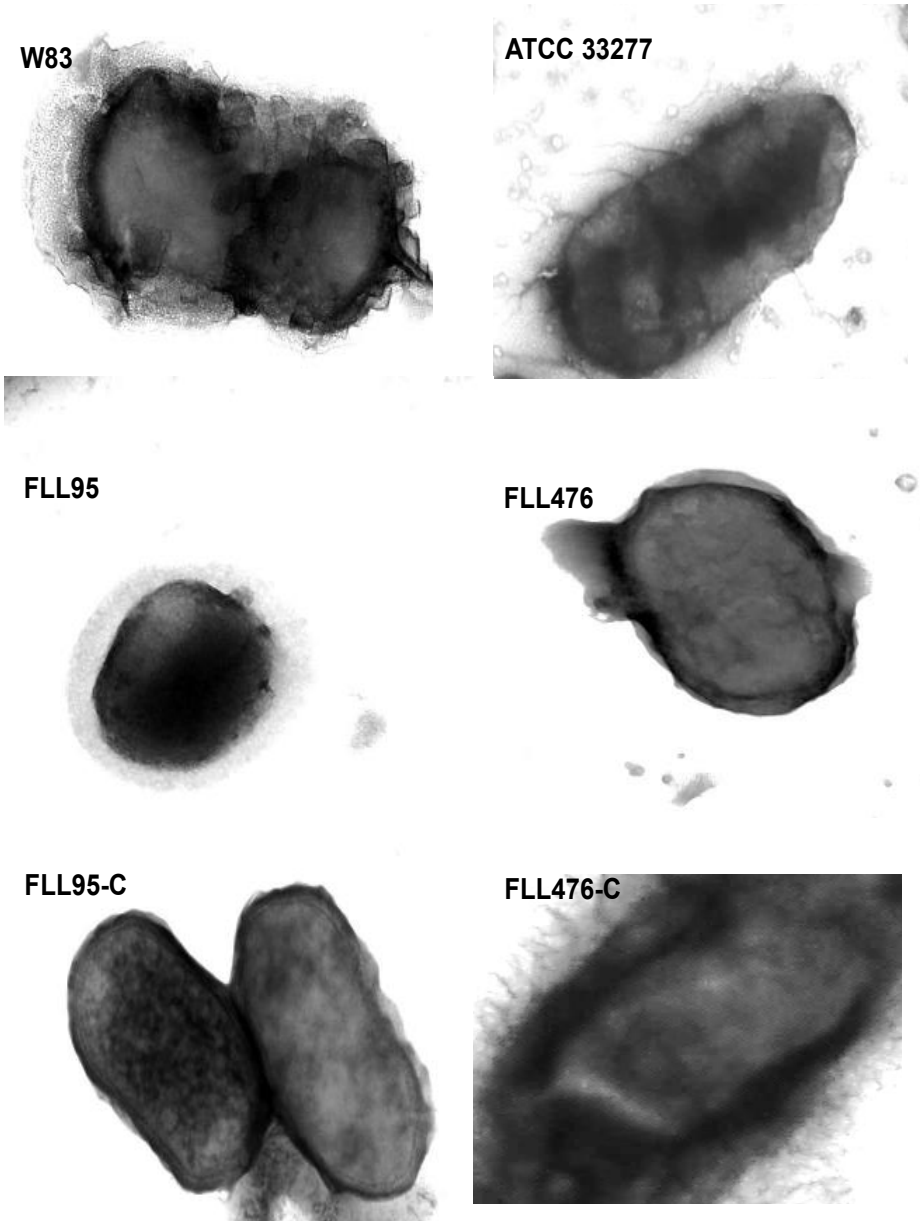


Figure 2.3. Electron micrograph showing changes in surface structures of *P. gingivalis* ATCC 33277 and W83. Bacterial cells grown to the log phase (OD_{600} of 0.7 -0.9) were processed for electron microscopic examination using formvar-carbon coated grids (500 mesh) and were examined using Philips Tecnai 12 TEM. Fimbriae were lacking in the *vimF* mutant FLL476 when compared with the wild ATCC33277 and the complemented strain FLL476C'. A thick glycocalyx along with vesicles and a well-defined outer membrane was observed in W83. FLL95 showed hazy outer membrane with reduced visicles. In the complemented strain FLL95C' the outer membrane morphology was restored.

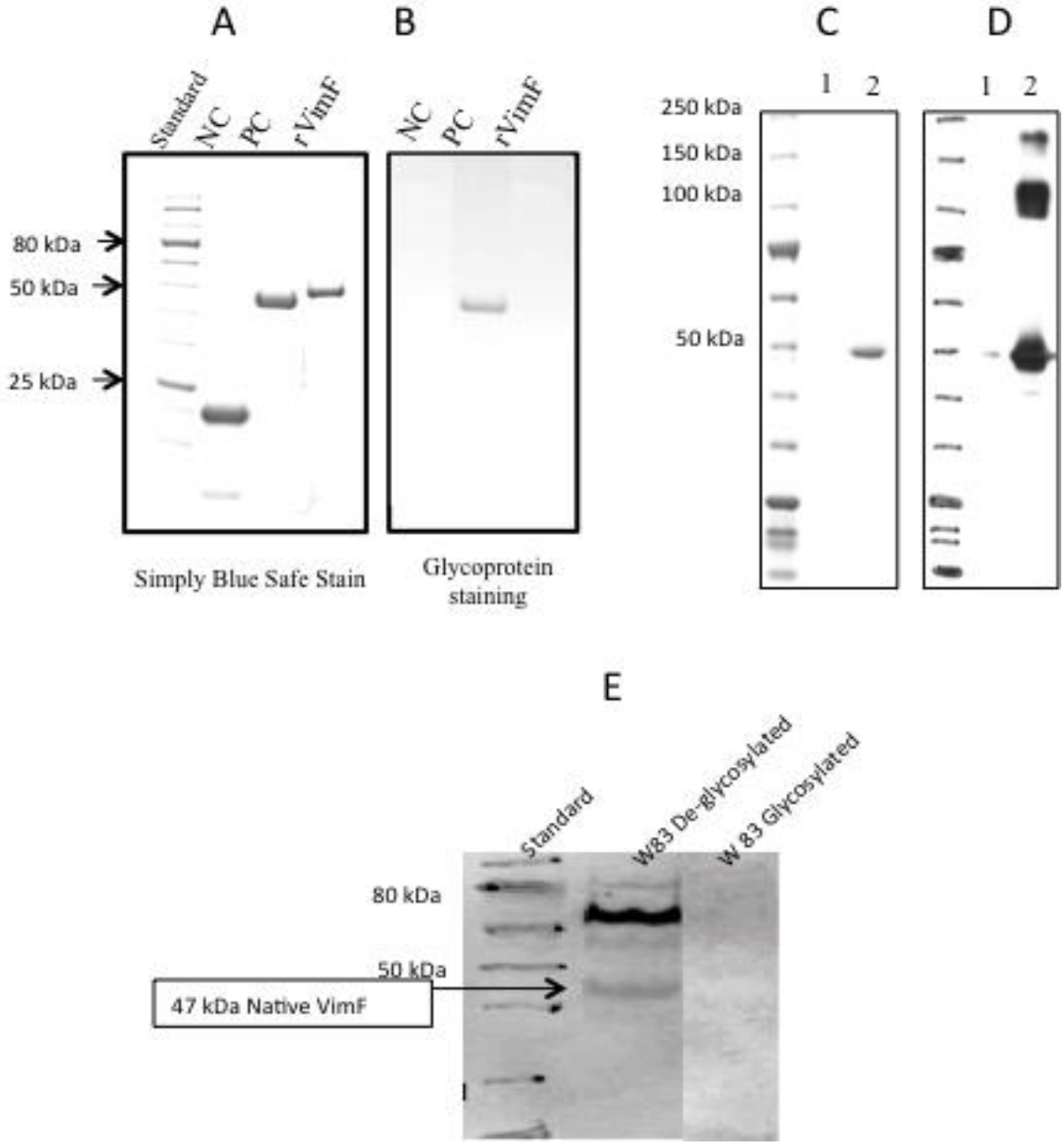


Figure. 2.4. 1D and 2D SDS-PAGE of rVimF. Purified rVimF was denatured in an LDS-containing buffer with DTT and heated for 10 min, and subjected to SDS-PAGE analysis. **A**. Simply Blue Safe stain of rVimF at 2 different concentrations: lane 1 – 0.4 µg and lane 2 – 1.2 µg. **B.** Western blot using anti-rVimF antibody against purified rVimF showed reacting bands at 50, 100 and 200 kDa. **C.** Simply blue safe stain of rVimF with horseradish peroxidase as positive (PC) and soybean trypsin inhibitor as negative (NC) controls for glycoproteins. **D.** Identical gel in panel C stained by periodic acid-Schiff (PAS) method for glycoproteins. **E.** Western blot using anti-rVimF showed a 47 kDa reactive band only when total proteins of W83 were deglycosylated and not with native (glycosylated) forms.

rVimF Shows Galactosyltransferase Activity

VimF is annotated as a putative glycosyltransferase type 1 (http://oralgen.lanl.gov). Thus the activity of rVimF was evaluated using a calorimetric assay [25] that exploits the lowering of pH resulting from the release of protons associated with glycosyltransferase activity. The change in pH is detected spectrophotometrically using a phenol red indicator. A calibration curve using known concentrations of HCl was used to establish the relationship between proton release and decrease in OD557 (Calibration curve). Commercially available UDP-galactose or UDP-glucose as donor substrate and, glucose, galactose, mannose, *N*-acetylglucosamine or *N*-acetylgalactosamine as acceptor substrate, was used to screen for rVimF glycosyltransferase activity. As shown in Fig. 2.5, the largest initial drop in OD557 was observed when UDP-galactose (Fig. 2.5A) was used as the donor substrate as compared to UDP-glucose as donor (Fig. 2.5B). Among the acceptor sugars used for UDP-galactose as donor, glucose followed by *N*-acetyglucosamine showed the lowest OD557 in the time course experiments suggesting their acceptor function in the presence of rVimF to these two sugars. Therefore for activity assays we chose to use *N*-acetylglucosamine as it is also the commonest acceptor used for commercially available β-1,4-galactosyltransferases (Sigma, St. Louis, MO) which we chose as positive control. Lysate from Top 10 cells was used as negative control.

Using UDP-galactose as donor and *N*-acetylglucosamine as acceptor the enzyme activity of rVimF was calculated and compared with a commercially available β-1, 4-galactosyltransferase. Fig. 2.6A shows a typical time trajectory of absorbance change corresponding to rVimF-catalyzed proton release (higher the proton release lower the OD557) in comparison to the positive and negative controls. Using the calibration curve,

rVimF-catalyzed proton concentration change corresponding to the absorbance change was calculated and plotted as a function of time (Fig. 2.6B). A linear regression was performed (R2 = 0.9242) and the slope was estimated to be 0. 1797. The enzyme activity of rVimF was calculated using the formula

$$0.1797 mmol/L/\min \times 1 \times 10^{-3} L \div 0.1 ml = 1.797 \mu mol/\min/ml$$
$$= 1.797 units/ml$$

Enzyme activity was defined as the amount of enzyme needed to produce 1 µmol of proton per minute. Using the same formula, enzyme activities of the positive and negative control was calculated to be 5.0 U/ml and 0.6 U/ml, respectively. Activity of positive control, commercially available β-1,4-Galactosyltransferase, estimated by our assay corresponds well with prescribed activity of > 0.6 U/ml suggested by the manufacturer (Sigma, St. Louis, MO).

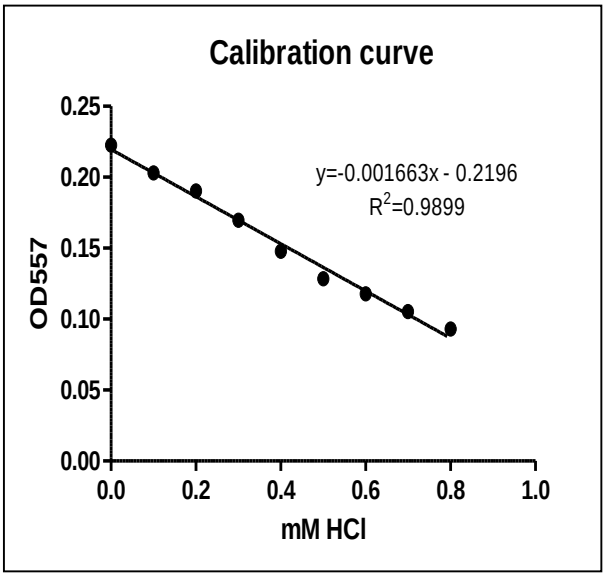


Fig. 2.4.1 Calibration Curve. Relationship between proton concentration and OD_{557}. Different concentrations of hydrochloric acid (0 - 0.8 mM) in mixture containing 2 mM phosphate, pH 8, 0.01 mM phenol red, 0.1 mM $MnCl_2$, and 100 µl of rVimF lysate were added and OD_{557} recorded.

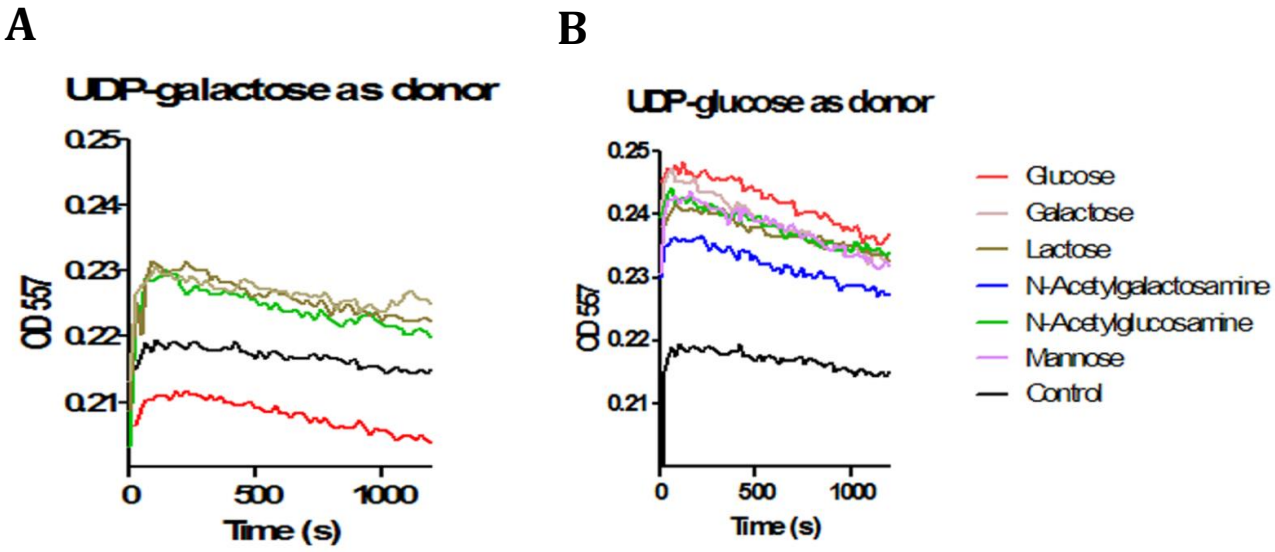


Figure 2.5. Screening for donor and acceptor substrates. Change of absorbance at 557 nm with time for **A**. UDP-galactose as donor and **B**. UDP- glucose as donor was plotted using various sugars as acceptors. Lysates (100 µl) of expressing rVimF was used as enzyme source and, lysates of Top 10 cells was used for negative control. The reaction mix contained 2mM phosphate, pH 8, 0.01mM phenol red, 0.1mM $MnCl_2$, and 10 mM acceptor sugars. A lower OD_{557} value was observed when UDP-galactose was used as the donor.

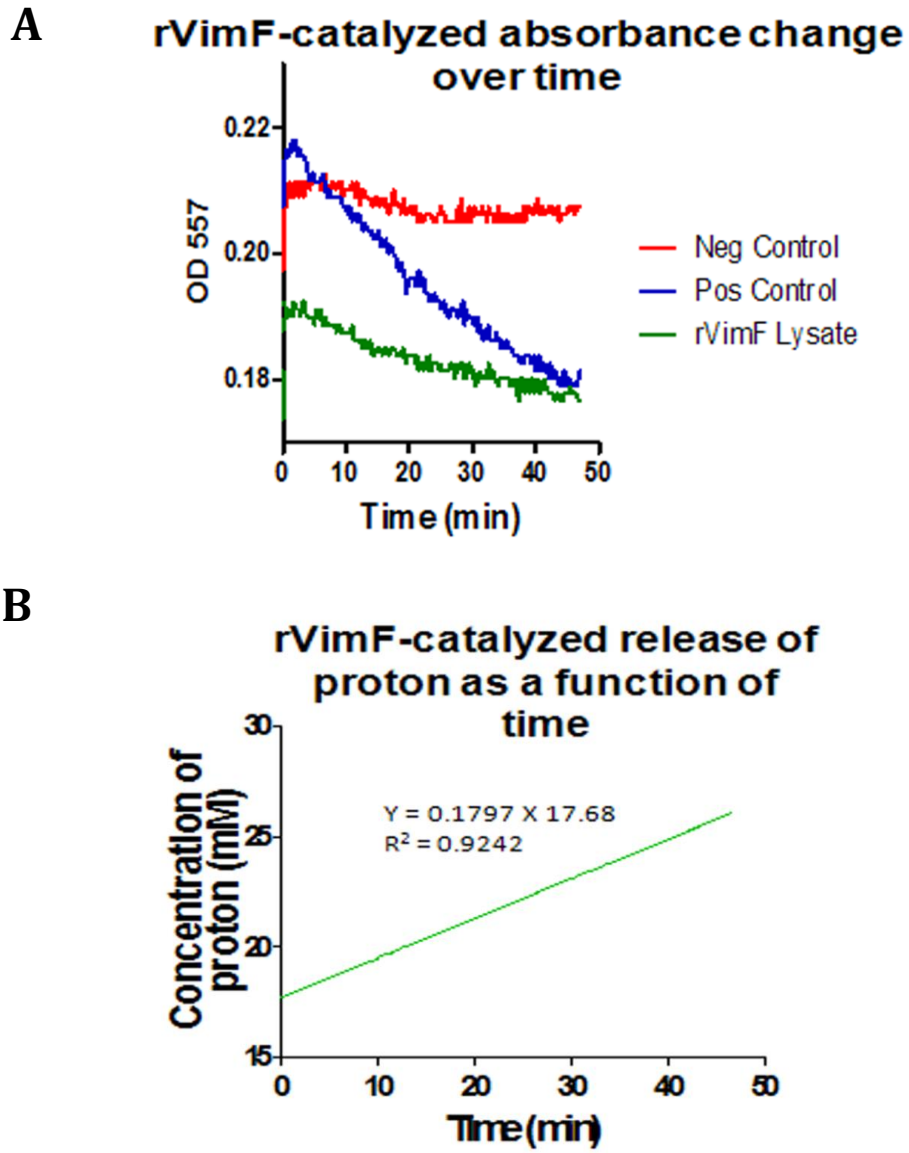


Figure 2.6. Galactosyltransferase activity of rVimF. Activity of rVimF compared with positive control (commercially available β- 1,4-galactosyltransferase) and Negative control (non-specific *E. coli* Top 10 lysate). 100 µl of whole cell lysates of *E. coli* containing pFLL477 expressing rVimF was added to reaction mix containing 2mM phosphate, pH 8, 0.01mM phenol red, 0.1mM $MnCl_2$, 10 mM *N*-acetylglucosamine and UDP-galactose was added to start the reaction. OD_{557} was measured for 50 minutes. **A**. Time course showing average of 3 independent assays. **B**. Activity of rVimF as a galactosyltransferase was calculated by converting change in OD_{557} to amount of proton release over time using the calibration curve in GraphPad Prizm 5 software.

FLL95 Gingipain Proenzyme Did Not Show Glycan Attachment

To further clarify the role of VimF in the glycosylation of gingipains, the presence of carbohydrates on the proenzyme gingipain species from FLL95 was determined using SDS-PAGE glycoprotein stain. As shown in Fig. 2.7A, no detectable band was observed in FLL95 in contrast to the gingipains from wild-type W83. For comparison a similar gel stained with SimplyBlue SafeStain (Fig. 2.7B) is shown. A more sensitive technique using methanolysis and silylation followed by GC-MS analysis of the TMS-methyl glycosides confirmed the absence of any detectable sugar moiety attached to the gingipain proenzyme species (data not shown). Taken together, these results suggest that the proenzyme species from the *vimF* defective mutant is devoid of any detectable glycan modification.

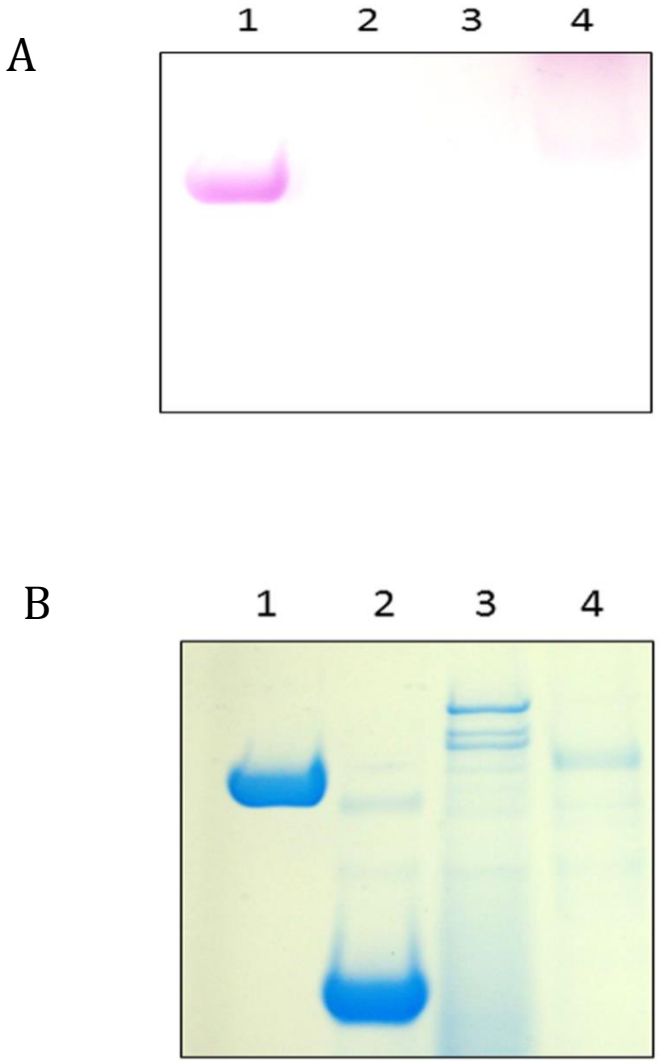
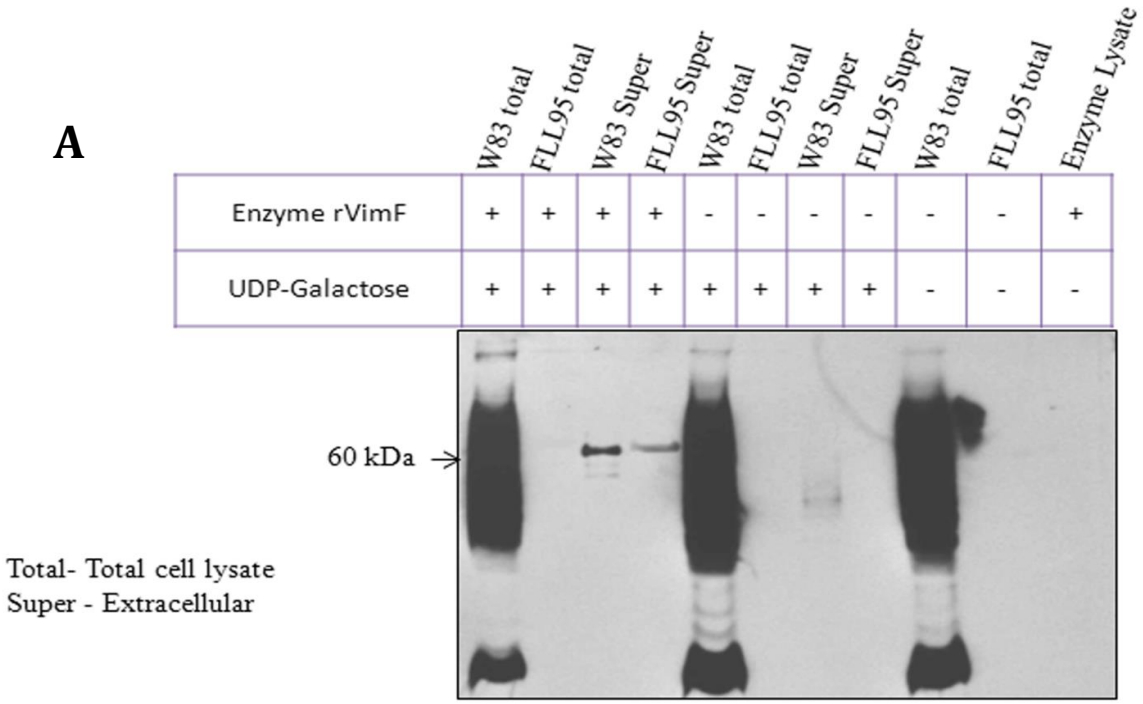


Figure 2.7. FLL95 Gingipain proenzyme is devoid of carbohydrate attachment. (A) Carbohydrate stain compared to **(B)** Simply Blue Stain of W83 catalytic domain and FLL95 proenzyme: lane 1, positive control using horseradish peroxidase provided by kit; lane 2, negative control using soybean trypsin inhibitor provided by kit; lane 3, FLL95 proenzyme; lane 4, W83 catalytic domain.

rVimF-Dependent Gingipain Glycosylation

In the *P. gingivalis vimF*-defective (FLL95) mutant the presence of the gingipain proenzyme species and their lack of immunoreactivity to mAb 1B5 suggest a glycosylation defect [12]. With the preference of rVimF to transfer galactose to *N*-acetylglucosamine, we evaluated its ability to transfer galactose to acceptor proteins in *P. gingivalis*. In the presence of rVimF and UDP-galactose, a 60 kDa band that immunoreacted with the glycan specific mAb 1B5 was observed in the extracellular fraction of FLL95 (Fig. 2.8A). This band was missing in the absence of UDP-galactose or when lysates of *E. coli* TOP 10 cells were used instead of rVimF in the reaction mixture (Fig. 2.8B). Using mass spectroscopy this 60 kDa band was identified as Rgp progingipain. A similar 60 kDa band in addition to a 45 kDa band was observed in the extracellular fraction of W83 only when rVimF was present in the reaction mixture.

A



B

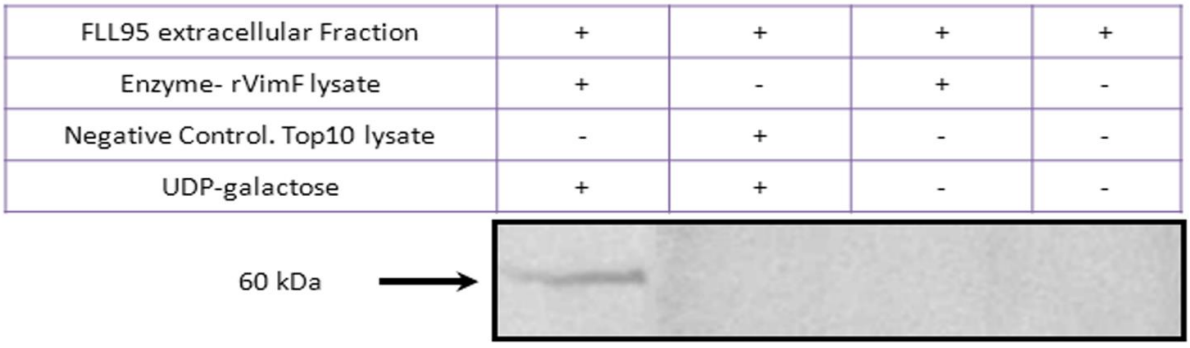


Figure 2.8. In-vitro galactosyltransferase assay. Total cell lysate or extracellular fractions from W83 and FLL95 were used as acceptor substrates, *E. coli* lysate carrying pFLL477 served as its enzyme source and UDP-galactose served as donor substrate. Western blots were probed with glycan specific mAb IB5. **A.** 60 kDa band appeared when both UDP-galactose and rVimF enzyme lysate were present. **B**. Using extracellular fractions of FLL95 as acceptor substrate a 60 kDa band was seen only when rVimF lysate and UDP-galactose were present. Negative control using Top 10 *E. coli* lysate did not show the 60 kDa band.

Table 2.1. Plasmids and Bacterial Strains Used

Plasmid/Strains	Phenotype and description	Reference
Plasmid		
pTrcHis2 TOPO	ampr, lacIq	Invitrogen
pFLL477	pTrcHis2 TOPO containing the *vimF* gene	This study
Bacterial Strains		
P. gingivalis		
W83	Wild type	(Abaibou, 2001)
FLL95	*vimF* mutant in W83	(Vanterpool, 2005)
FLL95C’	Complemented FLL95	This study
ATCC 33277	Wild type	
FLL476	*vimF* mutant in ATCC 33277	This study
FLL476C’	Complemented FLL476	This study
E. coli		
DH5α	F-Φ80dlacZΔ M15 Δ(lacZYA-argF) U169 recA1endA1 hsdR17 (rk-, mk+) phoA supE44 λ-thi-1 gyrA96 relA1	Invitrogen
Top10	F-mcrA Δ(mrr-hsdRMS-mcrBC) Φ80dlacZΔ M15 ΔlacX74 recA1 ara139Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG	Invitrogen

Table 2.2. Primers Used for the construction of mutants

Primer	Description	Sequence
P1	*vimF* forward	5'-ATGAAACGGGTACTCATCTTCGCCGA-3'
P2	*vimF* reverse	5'-GTTAGCGACGATCGATTCCAGTAGAC-3'
P3	*vimF*-1Kb upstream	5'-CGGGAAGAGAGTCCTTGCTTTTCAAAGCA-3'
P4	*vimF-erm* reverse	5'-GTCATTTATTCCTCCTAGTTAGTCATGGTCGATGG CCGTTTCGTAGTCG-3'
P5	*vimF-erm* forward	5'-TTCGTAGTACCTGGAGGGAATAATCATTCAGCAT CGTATCATGAAGTAC-3'
P6	*vimF*-1Kb downstream	5'-CTGCAGTACGGGCACGGTTG -3'
P7	*erm_F* forward	5'TGACTAACTAGGAGGAATAAATGACAAAAAAGAAATTGC CCG-3'
P8	*erm_F* reverse	5'-GATTATTCCCTCCAGGTACTACGAAGGATGAAATTTTTCA-3'
P9	*vimF* complement forward	5'-GAT CGG AAA GCA GCG CAA GCG ACT TAT-3'
P10	*vimF* complement reverse	5'-ATC TGT CGA ACT CCG GAC TGC CG-3'

Discussion

Proteolytic processing and glycosylation are important components in the maturation/activation/anchorage of the gingipains in *P. gingivalis* [38]. In this report we have used both genetic and biochemical approaches to further confirm the role of specific bacterial host factors in gingipain biogenesis. Several recent studies have identified the involvement of many nongingipain genes in this process [39–44]. In addition a conserved C-terminal domain (CTD) is essential for the secretion and attachment of the gingipains to the cell surface [45]. Collectively, an emerging picture from these studies suggests a complex process that is facilitated by the appropriate glycosylation of the gingipains.

Glycosylation is a recently identified post-translational modification of proteins in prokaryotes [19]. This process which involves the enzymatic attachment of a glycan moiety to a protein is known to influence biological properties like activity, solubility, folding, conformation, stability, half-life, and/or immunogenicity [46,47]. Since the initial report of the S-layer glycoprotein of archaea *Halobacterium halobium* (salinarum), some of the well-known examples of bacterial glycoproteins belong to genera *Campylobacter, Mycobacterium, Neisseriae, Pseudomonas, Chlamydiae, Escherichia* and *Porphyromonas* [7,19]. In *P. gingivalis*, a defect in several GTases including Rfa, UgdA, GtfA, GtfB have been shown to affect polysaccharide biosynthesis that have resulted in decreased gingipain activities [11,17,18]. VimF, which is annotated as a putative glycosyltransferase (www.oralgen.lanl.gov) has been shown to be involved in maturation of gingipains, hemolysis, hemagglutination and fibronectin cleavage similar to some of the other GTase-defective mutants previously described [12,48]. In this study, several of these phenotypic characteristics were confirmed in a different *P. gingivalis*

genetic background. These observations further support the vital role of VimF in the pathogenesis of *P. gingivalis*.

Compared to the wild-type, the growth rate and gingipain activity were reduced in the *vimF*-defective mutants in both genetic backgrounds. However, the gingipain activity in *P. gingivalis* FLL476 was reduced by approximately 60% compared to more than 90% reported in *P. gingivalis* FLL95 [12]. This observation could suggest the role of additional factor(s) that may be involved in the maturation/glycosylation of the gingipains. For example, a functional homologue of *vimF* or an alternate glycosyltransferase, which is more effective in the *P. gingivalis* 33277 genetic background could be responsible for the increased activity. There are multiple glycosyltransferases reported in *P. gingivalis* (http://www.cazy.org/), however the substrate specificity for several of these enzymes is unknown.

The invasive capacity of the *vimF*-defective isogenic mutant in multiple genetic backgrounds was reduced compared to the wild-type. This was not unexpected, given the cell surface alterations in the *vimF*-defective mutants. This alteration in cell surface is also thought to contribute for the increased biofilm formation observed in FLL476. Similar observations have been reported[16,49]. The role of fimbria in host cell invasion by *P. gingivalis* is well documented [50]. In the *vimF*-defective isogenic mutant, the phenotypic expression of fimbria appeared to be altered. In addition it is known that a defect in LPS biosynthesis in *P. gingivalis* can influence attachment of the gingipains to the cell surface, autoaggregation, and biofilm formation. These phenotypic properties are known to be associated with *P. gingivalis* invasive capacity.

We have observed that the His-tagged rVimF, a 50 kDa protein, is homotetrameric and, on 2D gel showed isoforms. Purified rVimF was observed as a single band (Fig. 2.4A). However, the same gel, when subjected to western blot analysis and probed with anti-rVimF antibody showed immune reactivity to three different bands corresponding to 50 kDa, 100 kDa and 200 kDa (Fig. 2.4B). This seemingly conflicting observation is possible as rVimF may not be completely denatured in the presence of 10% SDS. The multimeric rVimF forms are consistent with the 200 kDa band observed in a native gel. Isoforms observed on the 2D gel is likely due to post-translational modifications of the rVimF protein. The type of modification is under further investigation.

In *P. gingivalis*, the anti-rVimF antibody immunoreacted with multiple protein bands only after deglycosylation. The 47 kDa protein band is the expected size of the VimF product and likely suggests that it's a glycoprotein. The 60 and 80 kDa immunoreactive bands may have conserved domains that can cross react with the anti-rVimF antibody although we cannot rule out mutimeric forms of VimF. Further investigation of their identity and possible function is underway.

The *P. gingivalis vimF* gene encodes for a 47 kDa protein that has galactosyltransferase activity. This, possible multimeric protein, was demonstrated to have the ability to transfer UDP-galactose to *N*-acetylglucosamine. VimF, which may also be a glycoprotein, is suggested by these studies to play a specific role in gingipain glycosylation. In contrast to the *vimA*-defective mutant which only had the RgpB gingipain cell associated, or other GTase-defective mutants that are missing any cell-associated gingipain, the *vimF*-defective mutants had both cell and extracellular

associated inactive forms of the gingipains [43,44,51]. Throughout all the growth phases, no activation of the gingipains was observed. Variation in the glycosylation profile of the gingipains including the missing phosphorylated branched mannan was also noted [44,52] in the *vimF* mutants. In the presence of rVimF and UDP-galactose, a 60 kDa band identified as RgpB regained its missing phosphorylated branched mannan. This could imply that galactose is important for the addition of the glycan moiety carrying the phosphorylated branched mannan. The monosaccharide composition of the gingipains from *P. gingivalis* W50 is known to include arabinose, rhamnose, fucose, mannose, galactose, glucose, GalNAc, GlcNAc, and *N*-acetylneuraminic acid [13]. There also appears to be common steps in the synthesis of LPS and APS and the maturation of the gingipains [13,52,53]. A bioinformatic analysis of the Rgp-gingipains predicted two potential *O*-linked and 15 potential N-linked glycosylation sites for RgpA. This is in contrast to RgpB that was predicted to have 6 *N*-linked and no *O*-linked glycosylation sites. Reported elsewhere, the sugars in RgpA are thought to be present predominately in O-linked chains with the monosaccharide GalN(Ac) linked to Ser/Thr [13]. Most N-linked glycan chains occur via *N*-acetylglucosamine attached to asparagine and followed sequentially by hexoses such as galactose [54]. The results from this study suggest that galactose is vital for the growing glycan chain. Because the gingipains from the *vimF*-defective mutant were missing any detectable carbohydrate modifications, this raises questions regarding the monosaccharide protein link. While we cannot rule out an *N*-acetylglucosamine link, as observed in Haemophilus, it is likely that galactose can occupy these N-linked sites [55]. It is also unclear if the attachment of these glycans occurs either sequentially or en-block. Further, in these experiments it was unclear if the

gingipain could complete the maturation process and gain proteolytic activity. This is under further investigation in the laboratory.

Our observation that the inactive proenzyme species can be cell associated in the *vimF*-defective *P. gingivalis* strain raises questions on the specific glycosylation requirement for attachment. Further, based on previous reports, the posttranslational proteolytic processing of the gingipains involves multiple enzymes for their activation. RgpA and Kgp are known to require a surface located carboxypeptidase for activation in contrast to RgpB which is known to require a novel C-terminal signal peptidase [56,57]. It is clear from our studies that the appropriate glycosylation may be a prerequisite for proteolytic processing and the addition of galactose may occur early in the sequence.

In conclusion, we have presented in vitro evidence for posttranslational regulation of proteolytic activity in *P. gingivalis*. In vitro glycosyltransferase activity for rVimF was observed using UDP-galactose and N-acetylglucosamine as donor and acceptor substrates, respectively. Further, in the presence of the rVimF protein and UDP-galactose, the glycosylation of the RgpB proenzyme was restored. Together, these observations suggest the VimF glycoprotein is a galactosyltransferase that may be specific for gingipain glycosylation. Moreover, galatose is vital for the growing glycan chain. This model system will facilitate a more careful evaluation of glycosylation occurring in gingipain biogenesis in *P. gingivalis*. Components of this system could possibly be an important therapeutic target.

Acknowledgements

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CHAPTER THREE

A POLYCLONAL RABBIT ANTI-rVIMF MADE TO LOCALIZE VIMF IN *P. GINGIVALIS* SHOWED POTENTIAL TO STUDY HEMAGGLUTINATION DOMAIND OF GINGIPAINS

Abstract

Hemagglutination/adhesion domains play vital role in pathogenesis of *Porphyromonas gingivalis*, an important etiological agent of adult periodontitis. Among the three types of gingipains namely, RgpA, RgpB and Kgp, hemagglutination domains are present only in RgpA and Kgp. We have shown that the *vimF* was able to transfer galactose to gingipains. In order to localize VimF among sub cellular fractions we raised an anti-rVimF antibody. We observed that this polyclonal rabbit antibody was not able to react with native VimF from *P. gingivalis* W83. However, using less stringent western blotting conditions this antibody was able to react to a 40 and 42 kDa protein only from outer membrane and outer membrane vesicle proteins of *P. gingivalis* W83 and not from FLL95, the *vimF* mutant. Mass spectrometry done on these proteins identified the 40 kDa protein as Kgp and the 42 kDa band as RgpA. Further only a single immunoreactive band was observed from FLL374 and FLL372, the Kgp and RgpA mutants respectively. Taken together the ability of anti-rVimF antibody to react with hemagglutinin domains of RgpA and Kgp could be used to study hemagglutinin domains of gingipains. Further, a VimF

chimeric strain having an extra copy of *vimF* with a 6X His tag, was constructed to localize VimF. This strain could also be used to study the biology of native VimF.

Introduction

P. gingivalis, the primary pathogen implicated in adult periodontitis is able to produce, among others, potent protease called gingipains which, like any other protease, is produced in an inactive form and post translationally processed to become mature/active gingipains. Among the three types of gingipain produced by this organism, namely RgpA, RgpB and Kgp, RgpB lacks the hemagglutination/adhesin (HA) domains that are found between the catalytic and the C terminal domain in both RgpA and Kgp [1,2]. Figure 3.1 shows various domains of gingipains. The HA domains are important for growth of this bacterium as this domains, anchored to the bacterial LPS, mediate assimilation of hemin, an iron containing porphyrin which is a vital ingredient for survival of this organism [3]. Recent studies have shown that RgpA and Kgp have multiple HA domains. It is shown that RgpA consists of a N terminal signal peptide followed by pro-peptide and the catalytic proteinase domain (42-kDa) and hemagglutination/adhesion domains at the C terminus, named HGP44, HbR, HGP17, HGP27 (previously called RgpA44, RgpA15, RgpA17, RgpA27), respectively [1,4,5]. Kgp also consists of similar domains (Fig. 1). Interestingly, the hemagglutinin/adhesion domains are also encoded by the hemagglutinin gene *hagA* with similar C-terminal regions (HGP44 and HbR). Further it is shown that the HA domains contain repeated adhesion binding motifs mediating binding to host proteins including fibronectin,

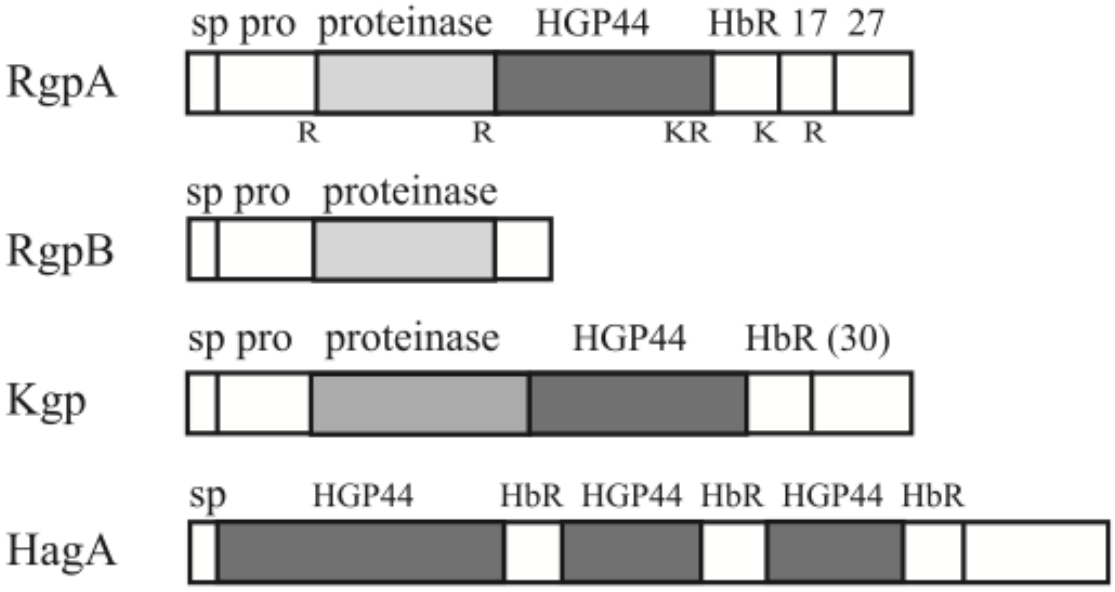

Figure 3.1. Gingipains and HagA Show Domain Homologies. RgpA and Kgp consist of signal peptide (sp), propeptide (pro), proteinase and adhesion domains (HGP44, HbR, HGP17, and HGP27). R and K indicate cleavage sites for Rgp and Kgp, respectively. RgpB lacks HGP44 and HbR domains. Reproduced from Sakai E et.al [5].

fibrinogen and type IV collagen [6]. The Hgp domain has been reported to adhere to collagen and fibronectin, which are intracellular matrix components [6]. Moreover, Rgp is linked to processing of fimbrilin and other surface proteins [7-9]. Although various studies have implicated HA domains for hemagglutinating activity [10,11], direct evidence of the molecule that exhibit hemagglutination and adhesion has not been obtained. Further, the steps involved in the processing of this HA domains are poorly understood.

In addition it was shown that RgpA-Kgp complexes (otherwise called high-molecular-weight gingipains) encoded by *rgpA* and *kgp* are major virulence factors in the pathogenicity of *P. gingivalis* W50 [12]. This observation has been verified as mutants of these genes had attenuated virulence in rat periodontitis models and, vaccines against these complexes were able to protect the rats against challenge [13,14]. Further studies have shown that these high molecular weight membrane associated mt-RgpAcat and HRgpA are glycosylated [15,16]. Although one study has shown the role of RgpB in glycosylation of these proteins, the amount of glycosylation seen in these proteins (14% - 30%) suggest role of other genes including glycosyltransferases that may be involved in processing of these forms of gingipains [17,18].

Glycosylation in bacteria is now a well-accepted post-translational modification and both N and O- glycosylations have been reported from bacteria [19]. While it is well accepted that glycosylation occurs in the endoplasmic reticulum and the Golgi of eukaryotes, in bacteria, which lack these organelles, it is not clear where glycosylation occurs [20]. However, starting from the first observation of the S-layer glycoproteins of Halobacterium halobium (salinarum), the list of bacterial glycoproteins has been growing

long [21]. Bacterial glycoproteins now includes gingipains of *P. gingivalis* in which about 14 – 30 % by weight is due to carbohydrates [17].

Although more than 20 glycosyltransferase genes are present in the genome of *P. gingivalis* W83, only a few have been characterized. All of the glycosyltransferases investigated from *P. gingivalis* so far (gtfA, gtfB, PG0106 and *vimF*), have been shown to be closely associated with pathogenic abilities. In the gtfB mutant there was complete loss of surface-associated gingipain proteinases, autoaggregation and biofilm formation due to defective O-LPS and A-LPS biosynthesis [22]. In the PG0106 mutant (which is closely related to *vimF* phylogenetically and involved in capsular biosynthesis) there was an increase in auto-aggregation and biofilm formation suggesting surface variations leading to this phenotype [23]. In gtfA mutant, the major fimbriae was not mature leading to decrease in auto aggregation and attachment to epithelial cells suggesting role in pathogenicity by regulating adhesion [24]. Finally, reduced autoaggregation, increased biofilm formation, reduced gingipain activity that were secreted, and abolition of hemagglutination activity were also observed in the *vimF* mutant of W83 [25] and ATCC 33277 (FLL95 and FLL476. Ref, 2nd chapter) suggest their role in pathogenicity. Although we have shown that VimF is involved in glycosylation of arginine specific gingipains, we were led to evaluate their role in glycosylation of other *P. gingivalis* proteins.

While evaluating the polyclonal rabbit antibody raised primarily against rVimF, we noted its inability to bind to native VimF unless it was first deglycosylated, suggesting differences in glycosylation machinery between and *P. gingivalis* despite the fact that rVimF produced from showed in-vitro enzymatic activity. In this chapter we

present our observations that under reduced stringency levels anti-rVimF antibody could cross-react with a 40, 42 and 80 kDa proteins seen only on outer membrane and outer membrane vesicle fractions of *P. gingivalis* W83 and not in the *vimF* mutant FLL95. Furthermore, mass spectrometric analysis of the reactive protein bands from various subcellular fractions of W83 and FLL95 were identified. In addition, we present the initial characterization of a *vimF* chimera (FLL479), which contained an extra copy of *vimF* at the *fimA* site along with a N-terminal 6X His tag. This strain was engineered to purify native VimF from *P. gingivalis* W83, ascertain its function as a glycosyltransferase and also to localize the VimF protein among *P. gingivalis* subcellular fractions.

Materials and Methods

Bioinformatic Analysis of VimF

Using the amino acid sequence retrieved from from Oralgen (http://www.oralgen.lanl.gov) the secondary structure prediction and modeling of protein was done using Modeler 9v8 and validated using WHATIF program [26,27]. The signal peptide and potential cleavage sites were predicted using both the neural network and hidden Markov model [28]. Bioedit (http://www.nbio.ncsu.edu/bioedit/bioedit.html), the online biological sequence alignment editor, was used to align amino acid and DNA sequences retrieved from Oralgen database. Phylogenetic relationship and distance was calculated using MEGA version4.0 and Kimura 2- parameter model, and clustering the neighbor-joining method was employed using boot strap values based on a thousand replicates [29,30].

Bacterial Growth Conditions

Strains of *P. gingivalis* used in this study listed in Table 3.1. were grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) supplemented with 5 μg/ml hemin, 0.5 μg/ml vitamin K and 0.1% cysteine. Five percent agar and 5% sheep blood were added to make blood agar (BA). Ten μg/ml of erythromycin was used to select resistant colonies. All cultures were incubated at 37oC in an anaerobic chamber (Coy Manufacturing, Ann Arbor, MI) with 80% nitrogen, 10% carbon dioxide and 10% hydrogen.

Cell Fractionation

The cytoplasmic, inner membrane (IM), outer membrane (OM), Vesicle (V) and particle free culture supernatant fractions were separated as previously reported with few modifications [31,32]. Briefly, cells grown to late log phase were centrifuged for 30 min, at 10,000g to separate cell pellet from the culture medium. Supernatant containing culture medium was then ultra-centrifuged at 100,000g for 60 min at 4oC. Resulting pellet was re-suspended in 10 mM Tris HCl (pH 7.4) and considered as the vesicle fraction (Ves), and the ammonium sulphate precipitated (80%) supernatant resuspended in 10 mM Tris HCl (pH 7.4) was considered as particle free supernatant containing the extracellular fraction (Ext). Cell pellet was washed thrice with 1X PBS and resuspended in PBS containing 0.1mM N-p-tosyl-L-lysine chloromethyl ketone (TLCK), 0.1 mM leupeptin, and 0.5 mM EDTA. Cells were then lysed using French press (American Instrument Company, Silver Spring, MD) in the presence of protease inhibitor by 5 passes. After removing the bacterial cell debris by centrifuging at 10,000g for 20 min, the supernatant

was ultra-centrifuged at 100,000g for 60 min at 4oC. While resulting supernatant was ammonium sulphate precipitated and considered as the cytosolic fraction (Cyto), the pellet was treated with 1% Triton-X-100 in PBS containing 20 mM MgCl2 for 30 min at 20oC and ultra-centrifuged at 100,000g for 60 min at 4oC. While the precipitate was considered as the outer membrane (OM) fraction the supernatant was concentrated as taken as inner membrane (IM).

SDS-PAGE and Western Blot

The different cell fractions were subjected electrophoresis, either in denaturing conditions in a standard 10% SDS-PAGE procedure or in the native conditions omitting SDS, reducing agent (DTT) and anti-oxidant in morpholineethanelsulfonic acid (MES) buffer as reported [25]. About 20 to 40 μg of protein was used for electrophoresis. Separated proteins were transferred on to nitrocellulose membrane using Semi-Dry Trans-blot (Bio-Rad) at 15V for 25 min. The blots were then probed with polyclonal rabbit anti-VimF antibody in either the stringent or less stringent conditions. For the less stringent conditions both blocking buffer and washing buffer contained 0.5 M NaCl instead of regular 1.3 M NaCl, 3% BSA was used in place of 5% milk, primary antibody was allowed to react for 1.5 hrs instead of 1 hr and washing after primary antibody was done only twice instead of four in the stringent conditions. Incubation with secondary antibody conjugated with HRP was for 30 minutes followed by two washing and finally, 5 minutes treatment with visualizing Chemiluminescence Reagent Plus kit (Perkin-Elmer Life Science, Boston, MA) followed by exposure to film were the same for both stringency conditions. Anti-RgpA antibody was used following stringent conditions.

Tryptic Digestion and Mass Spectrometry

SDS-PAGE separated protein bands and spots from 2D gels were excised and subjected to digestion with trypsin. The gel slices were first transferred to low retention epi vials (Fisher, Hampton, NH) and dehydrated using neat acetonitrile for 30 minutes. 20 µl of TCEP (tris(2-carboxyethyl)phosphine) was then added and incubated for 1 hr at 60oC. In the next step, 40 µl alkylating buffer (200 mM iodoacetamide) was added and incubated at room temperature for one hour. The gel slice was washed in 0.5 ml of neat acetonitrile and re-suspended in another 0.5 ml of neat acetonitrile to dehydrate. Next, digestion buffer containing mass spectrometry grade trypsin in 50 mM NH4HCO3 was added to attain a 1:20 to 1:50 enzyme/substrate ratio and incubated overnight at 40oC. Digestion was stopped using 10 µl of 10% formic acid. Digested peptides were extracted using standard Zip Tip (C18) technology (Millipore, Bedford, MA) according to manufacturer's protocol. MS analysis of extracted peptide was done as described elsewhere [33].

Localization of VimF in *P. gingivalis* by Immunogold Staining

Anti-VimF antibodies were purified and used at a working concentration of 1:1000. The colloidal gold conjugate (10 nm; Aurion) was diluted using incubation buffer (20 mM phosphate buffer, 150 mM NaCl pH 7.4, 0.2% BSA, 15 mM NaN3) to 1:1000. A checkerboard titration of primary antibody to immunogold conjugate was made to identify the optimal working concentration. Processed nickel grids were subjected to charging of the processed *P. gingivalis* strains for 1 h, then washed 20 times in 0.025 M Tris buffer (pH 7.4) and lightly blotted. Grids were subsequently blocked with 5% BSA in 0.025 M Tris buffer for 15 min at room temperature, then incubated in

diluted antibody–gold complex for 4 h at 37 °C. Staining was done using uranyl acetate (0.8 g uranyl acetate dissolved in 10 ml absolute ethanol) and freshly prepared lead citrate (10–40 mg lead citrate, 10 ml filtered water, 100 µl 10 M NaOH). Grids were immersed in uranyl acetate for 7 min at room temperature and then washed, once by immersion in 25% ethanol and twice by immersion in water. Grids were dried on filter paper for 10 min at room temperature and then floated on lead citrate drops for 5 min. Following two washes by immersion in 0.02 M NaOH and drying, grids were visualized using the Philips Tecnai FEI-12 TEM.

Construction of *vimF* Chimera

We used the PCR-based fusion of purified overlapping PCR amplified DNA fragments, a method described earlier [34], to insert *vimF* gene with an N terminal 6X-His tag and ermF which was amplified from pVA2198 into the *fimA* site. Primers used are listed in Table 3.2. Briefly, 1 kb fragments upstream and downstream of *fimA* were amplified using primers specifically designed to render amplicons with overhangs (Table 3.2). Four fragments namely, 1 Kb upstream of *fimA*, *vimF* with N-Terminal 6X His tag, *ermF* and 1 Kb downstream of *fimA* were amplified using primers designed to have overhangs complementary to adjacent oligomers. After purification these fragments were mixed in a reaction and subjected to 25 cycles of renaturing and annealing in the presence of PCR reaction mix without primers. Subsequently, one microliter of the sample was used in a PCR reaction using only P1 and R4 to get the fusion. Electrophoresis was used to ascertain the 3.5 Kd size of fused fragments. Fused PCR fragments were electroporated into *P. gingivalis* grown to the log phase as previously described [35].

Cells were then plated on to BHI agar containing 10 μg/ml erythromycin and incubated at 37oC for seven days. Colonies containing the correct gene replacement, as confirmed by colony PCR and DNA sequencing, were selected for further analysis.

Results

Structure Analysis of VimF

Bioinformatics analysis of VimF revealed beta sheets sandwiched by alpha helices that face each other, a structure consistent with glycosyltransferases. Figure 3.2 shows the following observations: An N-terminal signal peptide suggestive of the secretory nature of this protein, potential cleavage site between position 16 and 17 at the N terminal end suggestive of N terminal cleavage, two stretches of signature peptides (196 - 213 and 244 - 261) spanning the conserved structural motif were noted. The structure of the protein showed (Fig. 3.3) no membrane helix that suggests its localization on membrane or vesicles. Further a C terminal peptide suggestive of site of attachment to other proteins was noted. Phylogenetic analysis of all the glycosyltransferase-I in *P. gingivalis* W83 genome (Figure 3.4) revealed homology between VimF and PG0106, a glycosyltransferase linked to capsular polysaccharide synthesis [23]. Interestingly this protein is predicted to localize in the cytoplasm (www.stdgen.lanl.gov).

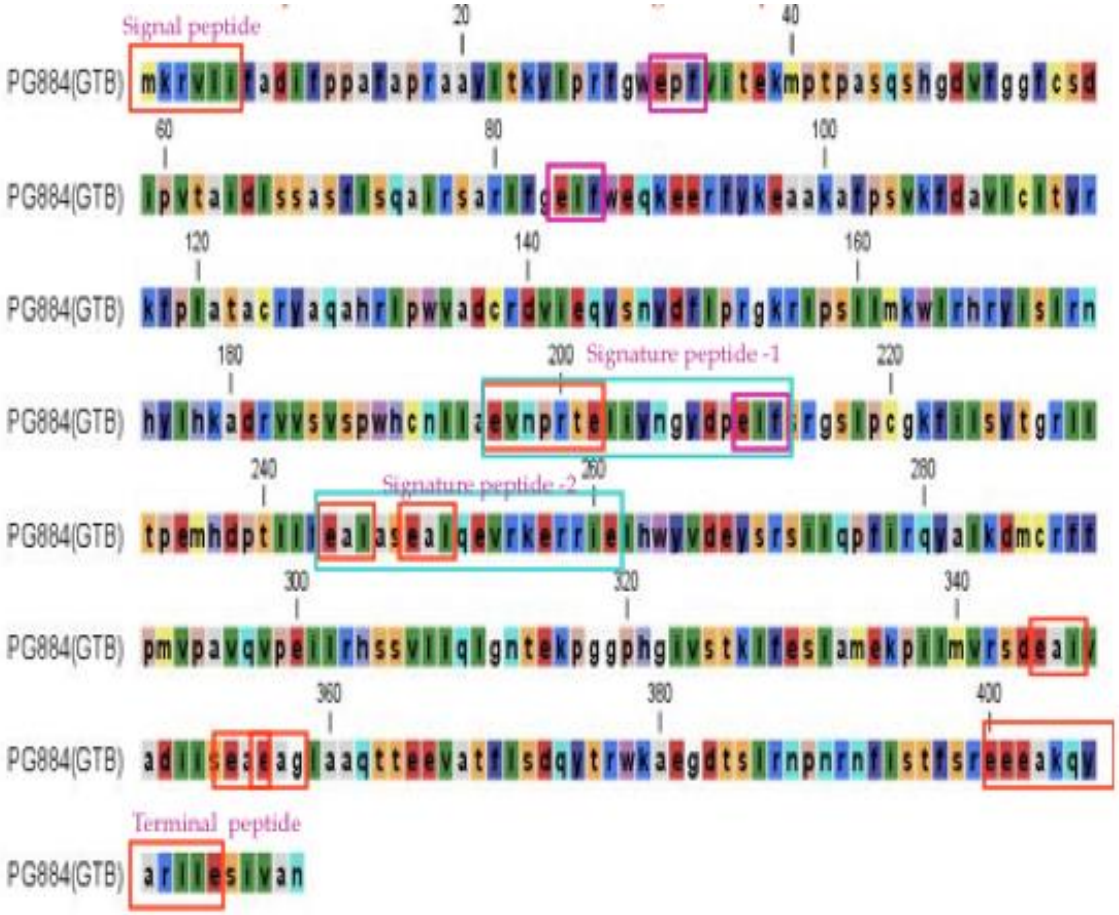

Figure 3.2. Amino Acid Sequence of VimF Showing Unique Signatures Peptides

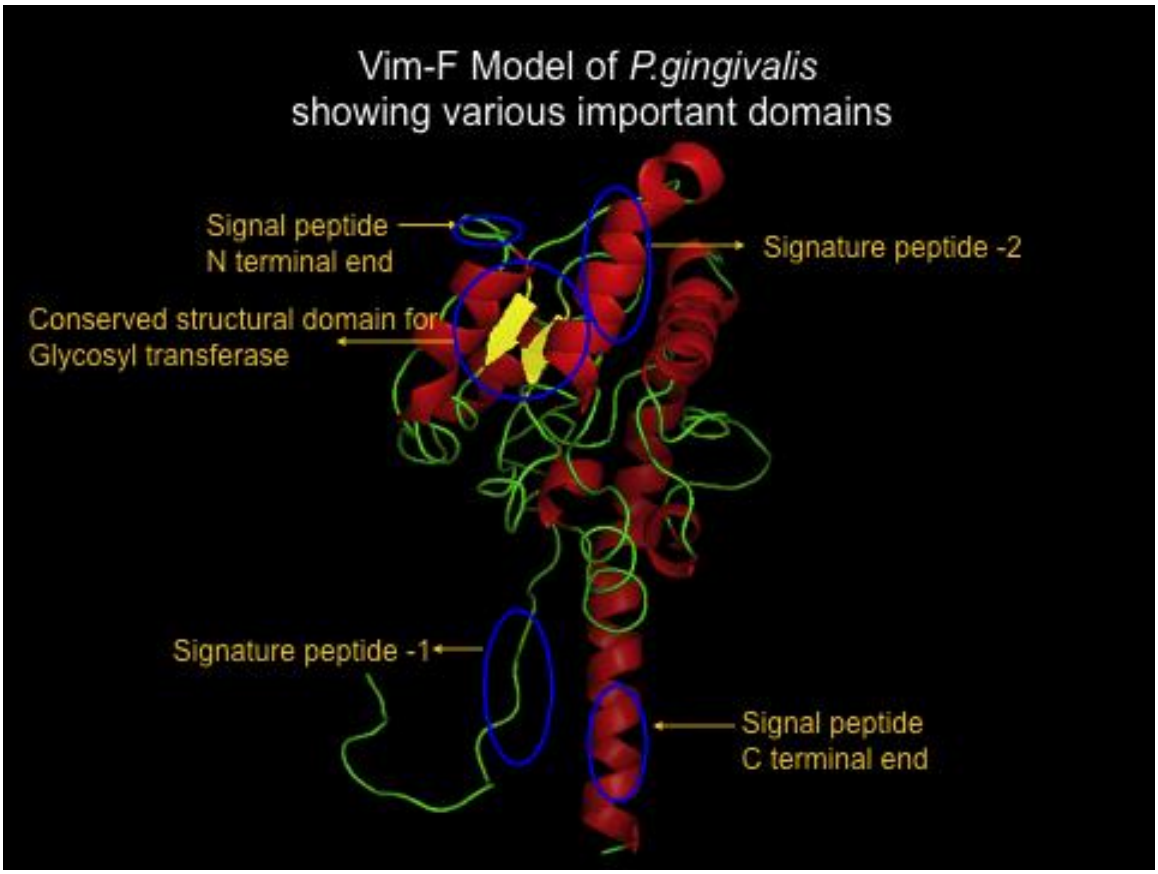

Figure 3.3. Model of VimF Showing Signature Peptides and Domains.

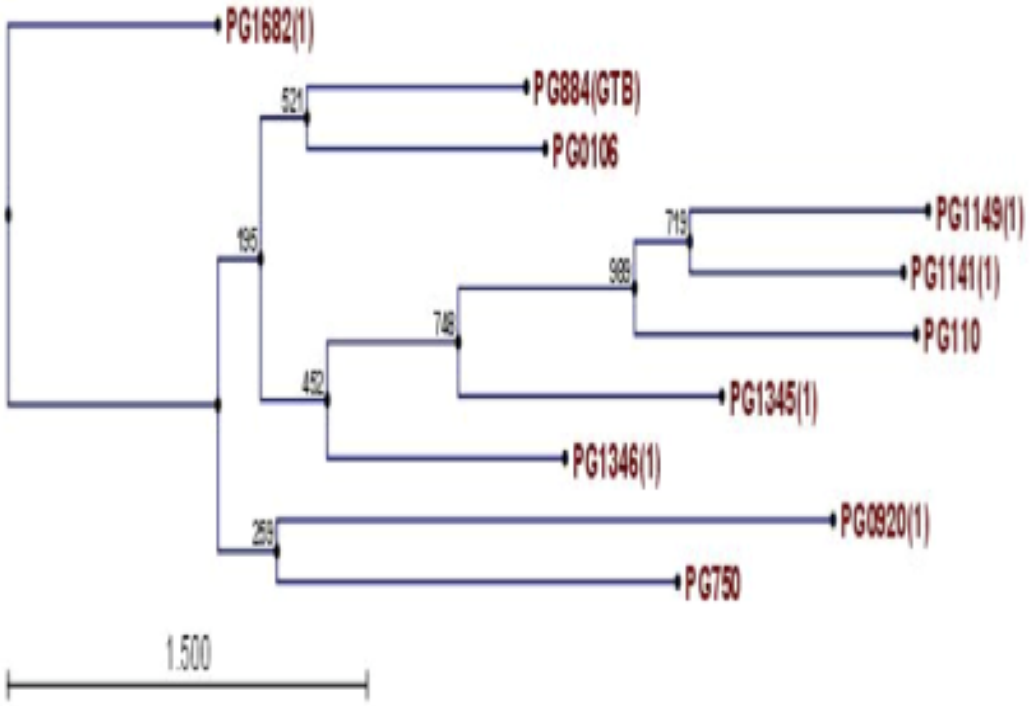

Figure 3.4. Phylogenetic Analysis of *P. gingivalis* Glycosyltransferase I Showed That *vimF* (PG0884) had Highest Homology to PG0106.

Anti-rVimF Antibody Did Not React With Native VimF

Polyclonal rabbit antibody raised against recombinant VimF was evaluated for reactivity with total cell lysates of W83. Figure 3.5 A and B shows the ability anit-rVimF antibody to react specifically with rVimF and its inability to react with any proteins from total cell lysates of *P. gingivalis* W83. However, as reported in chapter two, we have observed a 50 kDa reactive band when deglycosylated proteins of W83 whole cell lysates were immunoreacted with anti-rVimF antibody. When less stringent western blotting conditions was used, both pre-immune (Fig. 3.5C) and immune serum showed identical immunoreactive protein bands against *P. gingivalis* subcellular fractions although the bands appeared less prominent in the pre-immune serum.

Rabbit Anti-rVimF Reacts With 40, 42, 80 and 17 kDa Protein Bands in Denaturing Conditions

Figure 3.6 show the ability of anti-rVimF antibody to react with faint 17 kDa and 29 kDa proteins, well-defined 40 and 42 kDa proteins, and a diffuse well defined 80 kDa protein only from outer membrane and outer membrane vesicle fractions of W83 and not from FLL95. When FLL95 absorbed anti-rVimF was used in western blot the 29 kDa band seem to disappear. In addition subcellular fractions when electrophoresed under non-reducing conditions resulted in a single band around the 250 kDa which reacted with anti-rVimF in the western blot. Mass spectrometric analysis of the 40 and 42 kDa protein identified these proteins to be Kgp (PG1844) and RgpA (PG2024).

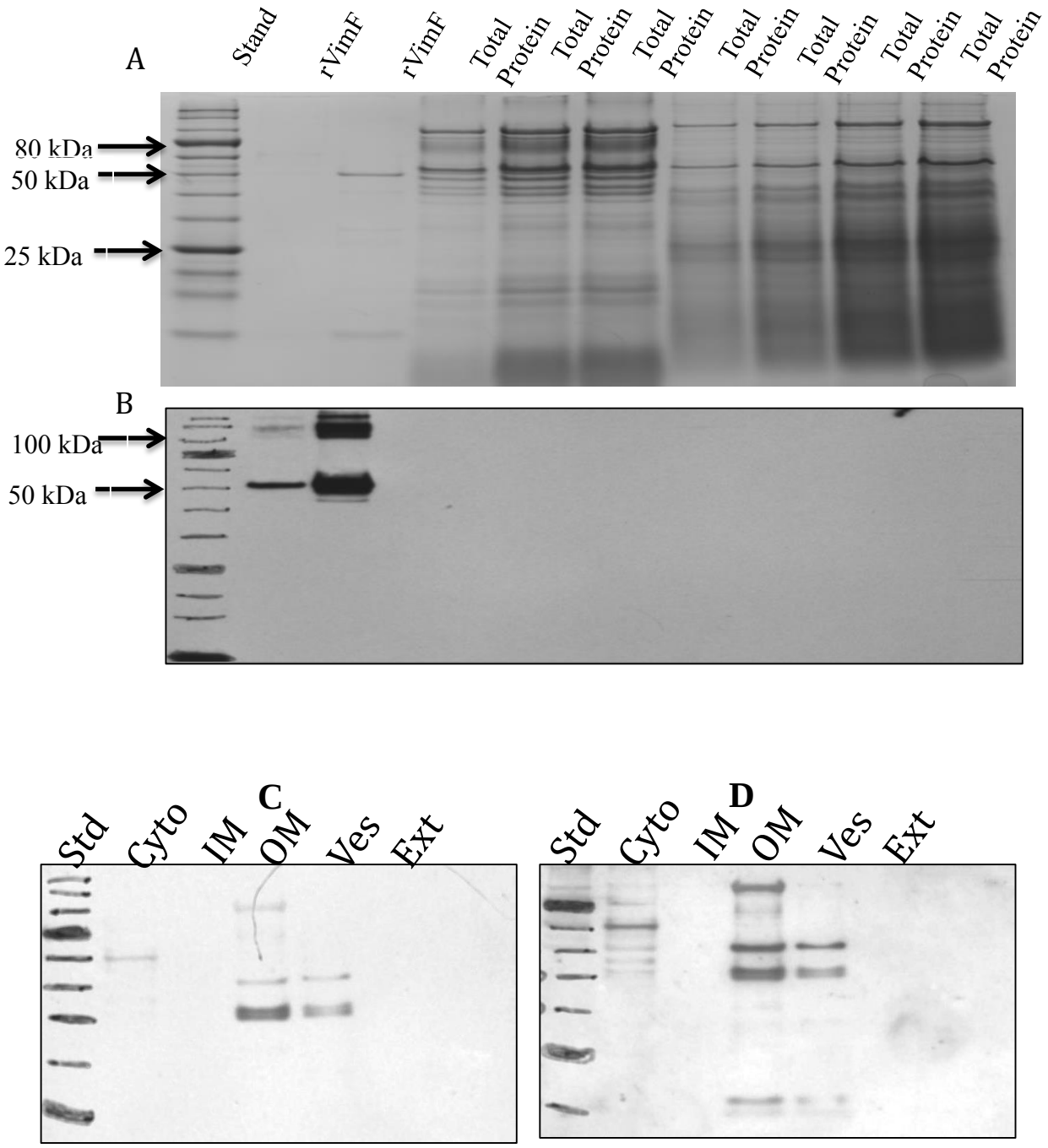

Figure 3.5. Under Stringent Conditions Anti-rVimF Antibody That Immunoreacted With rVimF Did Not React With Proteins from W83. **A.** SDA-PAGE of rVimF, total cell lysates of W83 and FLL95 at different concentrations of protein. **B.** Western using anti-rVimF antibody. Western using preimmune serum **C.** and Immune serum **D**. reacted with subcellular fractions of W83.

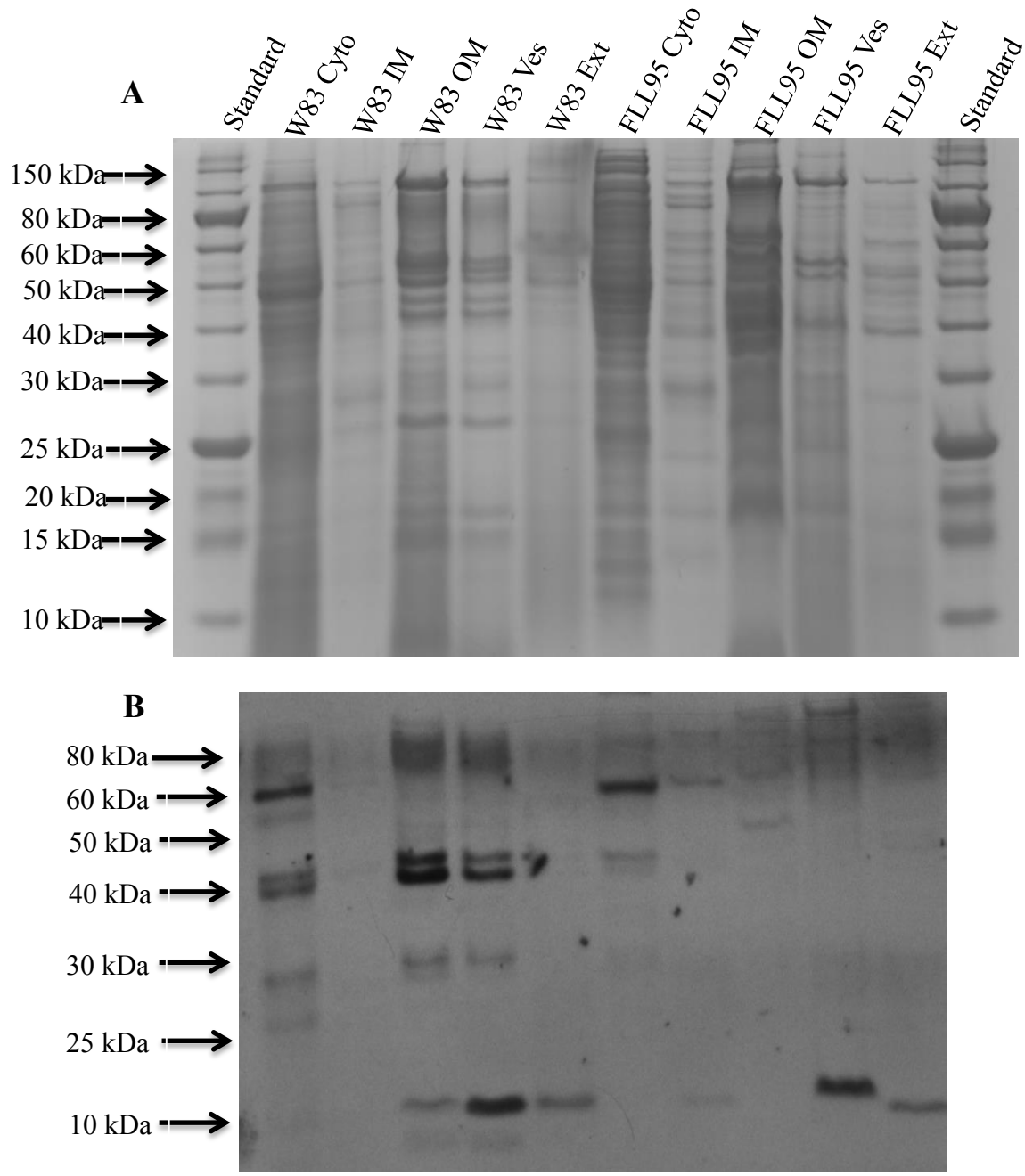

Figure 3.6. Anti-rVimF Reacted With 40 and 42 kDa Bands in Less Stringent Conditions. Various sub-cellular fractions: Cyto= cytoplasm, IM = inner membrane, OM=outer membrane, Ves=Vesicle and Ext=extracellular were subject to **A.** SDS-PAGE and **B.** blotted and probed with anti-rVimF antibodies at less stringent conditions showing the 40 and 42 kDa band from outer membrane and Vesicle fraction of *P. gingivalis* W83.

A Single Band Was Observed From *kgp* and *rgpA* Mutants

To further confirm the ability of anti-rVimF antibody to react with hemagglutinin domains of RgpA and Kgp we repeated the western blot using the anti-rVimF antibody against subcellular fractions of FLL374 and FLL372, the Kgp and RgpA mutant respectively. Figure 3.7A shows SDS-PAGE of subcellular fraction of the mutants. Only a single 42 kDa immunoreactive band was observed from outer membrane and vesicle fractions of FLL374 (Fig. 3.7B), the *kgp* mutant. Similarly, a single 40 kDa immunoreactive band was also observed from outer membrane and vesicle fractions of FLL372 (Fig. 3.7B), the *rgpA* mutant. These observations highlight the ability of antibody raised against rVimF to immunoreact with the 40 and 42 kDa hemagglutinin domains of *P. gingivalis* W83.

Immune Electron Microscopy (IEM) Using Anti-rVimF Antibody

Immunogold staining (Fig. 3.8) showed attachment of gold particles to the external surface of W83 especially surrounding the secreted vesicular structures during log phase. However, attachment of antibody tagged gold nanoparticles was not identified in FLL95 mutant. It is noteworthy that FLL95 did not produce any significant secretory vesicles. We also noted immunotagged gold nanoparticles only to wild type W83 cell suggesting the role of VimF glycosyltransferase in matured, secreted gingipain vesicles.

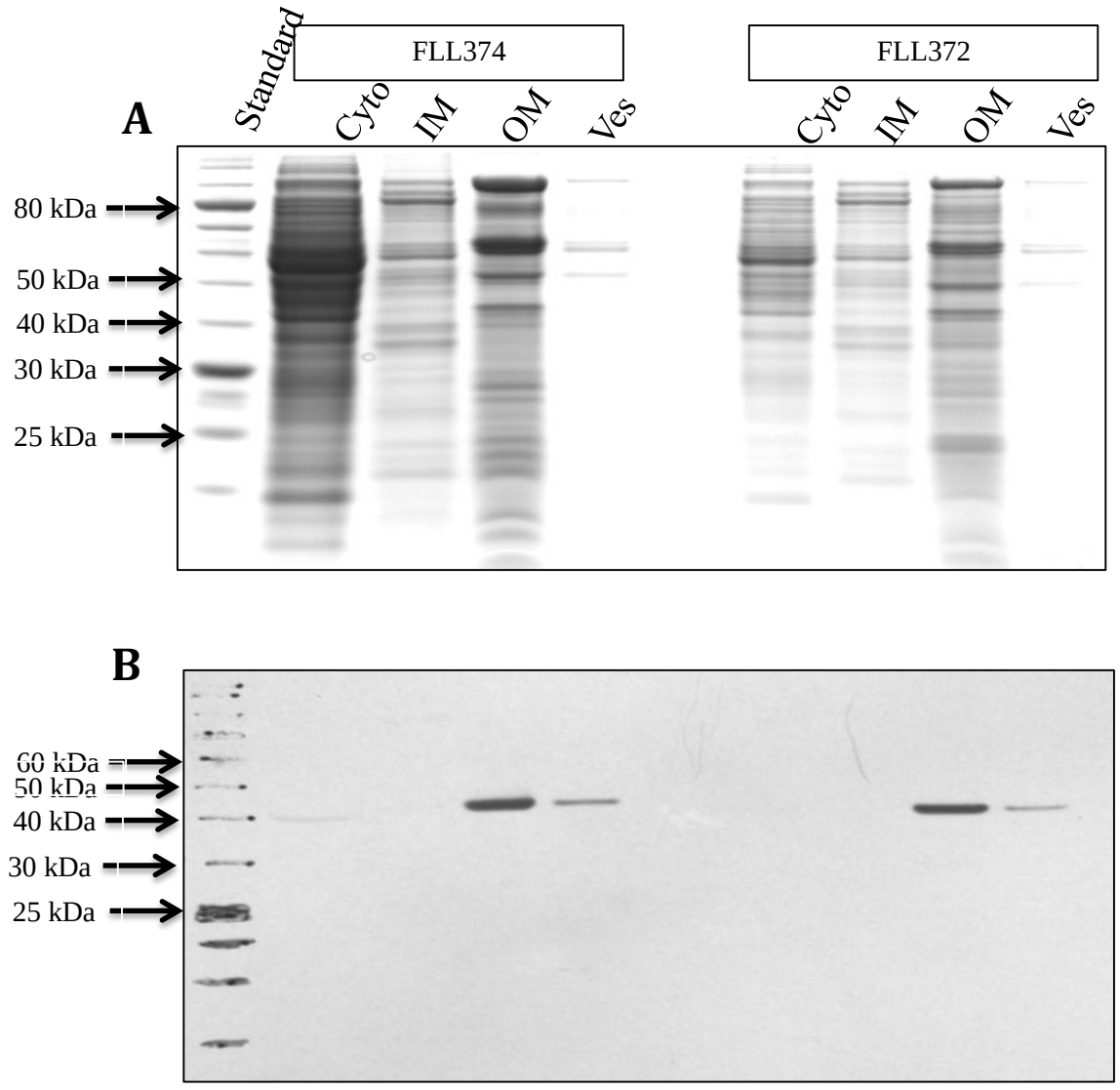

Figure 3.7. The *kgp* and *rgpA* Mutants Reacted With Single Band Near the 40 kDa Region. Western blot of sub-cellular fractions of *kgp* and *rgpA* mutants (FLL374 and FLL372 respectively) probed with anti-rVimF antibody showed single band from the outer membrane and vesicle fractions. **A.** SDS-PAGE gel of cytosolic (Cyto), inner membrane (IM), outer membrane (OM) and vesicle (Ves) fractions of *kgp* and *rgpA* mutants.

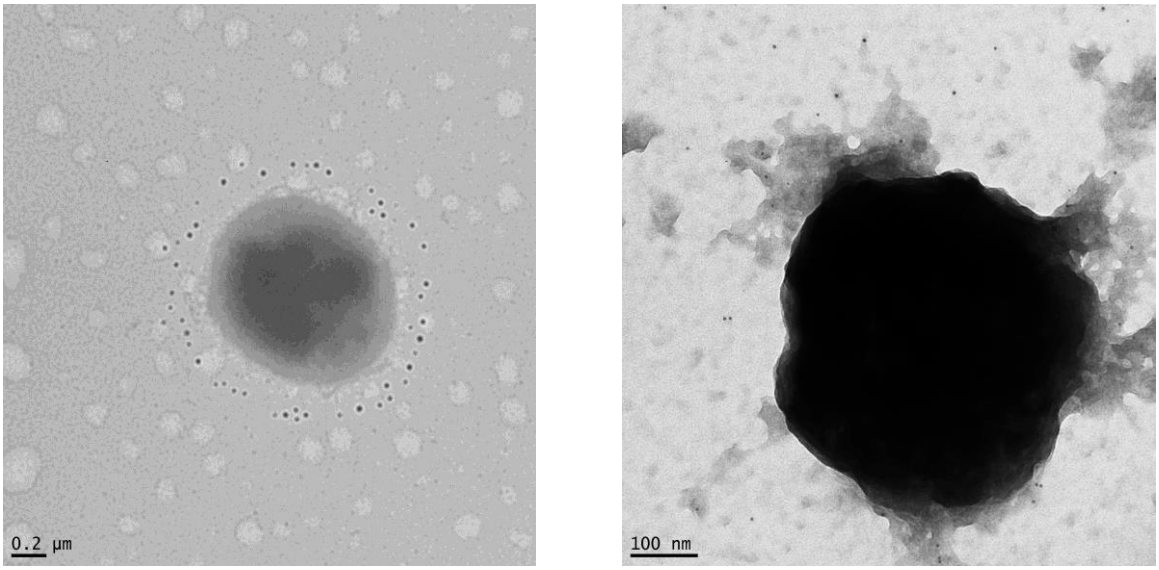

Figure 3.8. Immune Electron Microscopy Using Anti-rVimF Antibody Showing Immunogold Particles Attached to Vesicles of W83 but Absent in FLL95, the *vimF* mutant.

Construction of FLL479, the VimF Chimera

The 1 Kb upstream of *fimA, vimF* with N-terminal His tag, *ermF* and 1 Kb down stream of *fimA* were amplified separately in different PCR reactions (Fig. 3.9A). After purification the four fragments were mixed and subjected to 25 cycles of denaturing and annealing steps in a thermal cycler. Subsequently one microliter of this mix was used for PCR amplification in the presence of primers P1 and R4. As shown in Fig. 3.9B, the fusion fragment (3.5 Kb) was purified and electroporated into *P. gingivalis* W83 and incubated. Colonies were screened for the 3.5 Kb insert using primer P1 and R4 (Fig. 3.9C) and one colony containing this insert was called FLL479 and chosen for further evaluation. DNA sequencing confirmed the successful insertion of the purified construct into the *fimA* site.

Growth Characteristics, Gingipain, Biofilm and Hemagglutination Assays of FLL479

Similar to *P. gingivalis* W83, colonies of FLL479 were black pigmented and hemolytic on blood agar. Growth characteristic, proteolytic ability, and hemagglutination activity of FLL479 were also measured. Although growth curve (Fig. 3.10 A) and Rgp activity (Fig. 3.10 B) was similar to the wild type W83, a small increase in the Kgp activity (Fig. 3.10 B) was observed. Further, an increase in hemagglutination titer was observed for the VimF chimeric strain from various repeated measurements (Fig. 3.10 C).

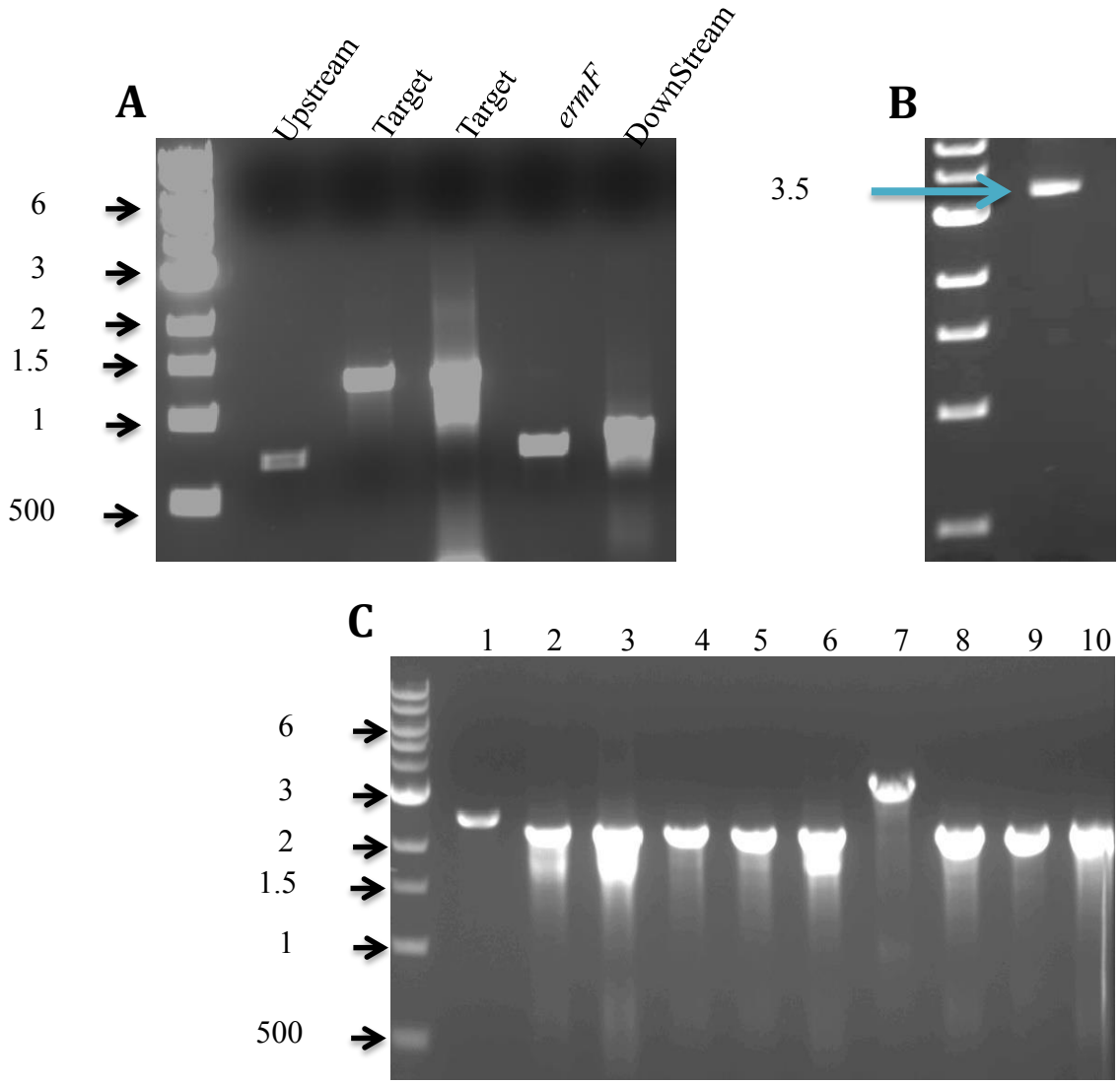

Figure 3.9. Construction of *vimF* Chimera. **A.** About 1 kb **upstream** and **downstream** of *fimA*, **target** containing *vimF* with N-terminal His tag and *ermF* were amplified in separate PCR reactions. **B**. Shows the fusion of the four fragments resulting in the 3.5 kb fragment. **C.** Screening of colonies for the insert showed one colony (lane7) containing the correct insertion.

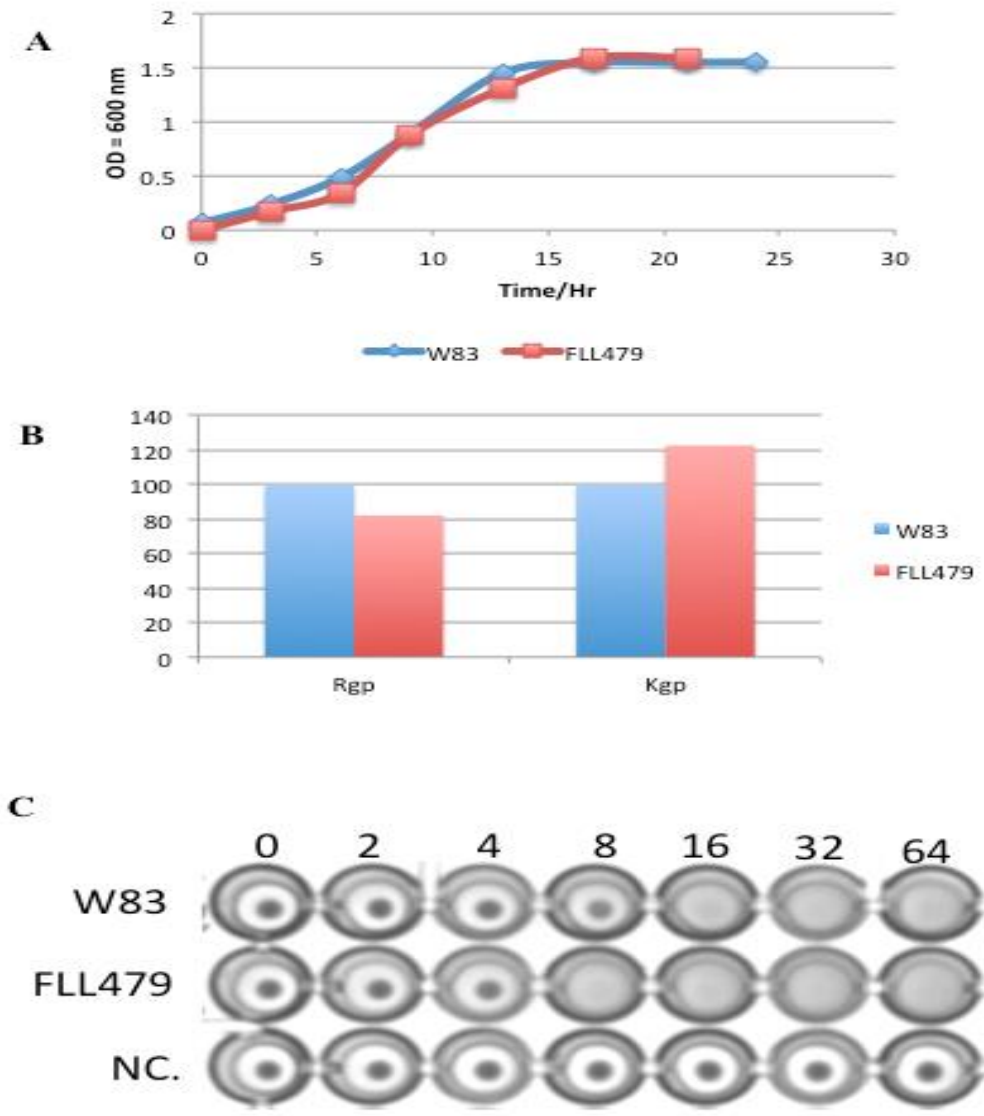

Figure 3.10. Characterization of FLL479, the VimF Chimera: **A. Growth curve –** OD_{600} was measured at intervals of time over a period of 24 hours. **B**. **Gingipain assay** – Rgp and Kgp activity were measured in whole cell cultrures grown to stationary phase. Gingipain activities of W83 were normalized to 100% to compare with the chimera. **C**. **Hemagglutination assay** – Serially diluted cells were incubated with sheep RBC in a round-bottom microtiter plates for 3 hours at 4^{o}C.

Table 3.1. Strains of *P. gingivalis* and Plasmid Used

Strain/plasmid	Phenotype and description	Source
W83	Wild type	[35]
FLL95	VimF-defective	[25]
FLL374	Kgp-defective	This study
FLL372	*rgpA*-defective	This study
FLL479	*vimF*-chimera	This study
pVA2198	*Spr, ermF-ermAM*	[8]

Table 3.2. Oligoneucleotide Primers Used for the Construction of FLL479

Primer	Sequence (5’ – 3’)
P1	ACAAGTACCCCATCCTAAAAAGTCGA
R1	GTTTATGATGATGATGATGATGCATCTCGTTTTTGTTTTAAAAGTG
P2	ATG CAT CAT CAT CAT CAT CAT AAA CGG GTA CTC ATC TTC GC
R2	CCATTTATTCCTCCTAGTTAGTCATTAGTTAGCGACGATCGATTCCAG
P3	TGACTAACTAGGAGGAATAAATGGAGAAAAAAATCACTGGAT
R3	GATTATTCCCTCCAGGTATTACGCCCCGCCCTGCCACT
P3	TACCTGGAGGGAATAATCTCGACCCGTCAAACGACTAAAAA
R4	GTCTGGGGCCATTAGCTTCCAG

Discussion

Bioinformatic analysis predicted VimF to be a glycosyltransferase-I with a B type fold. Although bioinformatics analysis show N terminal signal peptide motif suggestive of protein export through the conventional pathway, our earlier studies expressing the VimF protein in did not show the protein secreted, contrarily it was seen in the cytoplasm. Also it is noteworthy that VimF expressed in showed different posttranslational modification since an antibody raised against rVimF failed to react with native VimF from *P. gingivalis*. The closest phylogenetic neighbor PG0106 has been implicated in the synthesis of capsular polysaccharide [23]. This further stresses the role of glycosyltransferases in cell surface structures. However, further investigations are needed to implicate the role of native VimF in cell surface modification. In addition it will be worthwhile to investigate interacting protein partners, which will underline the involvement of VimF in cell surface biogenesis.

Although a polyclonal antibody raised against rVimF reacted well with rVimF from, it could not react with any protein from total cell lysates of *P. gingivalis* W83. However, the fact that reduced stringent immunoblotting conditions were able to react with hemagglutinin domains of Kgp and RgpA, suggest either a similarity between rVimF and the hemagglutinin domains of these proteins or a non-specific rabbit antibody to these domains. The later is probably the case as sequence alignment revealed no similarity between hemagglutinin domains and VimF, and, both pre immune and immune serum reacted with these protein bands. Also, it is possible that anti hemagglutinin antibodies can be made by animals as they are naturally exposed to various viruses that use hemagglutinins as target for attachment to host cells thus gaining access into the host.

Evaluation of anti-rVimF antibody against cytoplasmic and extracellular fractions revealed their ability to immunoreact with Kgp and RgpA domains. This was further confirmed as from *rgpA* defective mutant (FLL372) only the 40 kDa Kgp reacted with anti-rVimF and, from the kgp defective mutant (FLL374) only the 42 kDa RgpA immunoreacted with anti-rVimF antibody.

Owing to the cross-reactivity of anti-rVimF to HA domains in less stringent western blotting conditions it became difficult to draw clear conclusions of our results from the immune electron microscopy experiments. We observed strong reaction of anti-rVimF conjugated gold particles with vesicles from W83. These experiments, however, were done under routine IEM conditions. Given our observation that anti-rVimF antibody cross-reacts with HA domains, it is possible that we are observing the same cross reactivity.

Alternate ways to localize VimF have to be attempted as anti-rVimF antibody showed non-specific cross-reactivity with other proteins. Given our observation (chapter 2) that deglycosylated protein fractions could react with anti-rVimF in the stringent western blotting conditions, we will test the reactivity of the antibody with deglycosylated subcellular fractions of W83 and compare it with the FLL95. Further, we will use FLL479, the VimF chimera, which has an extra copy of the VimF in the *fimA* site to these localization studies. Additionally as this strain contains a 6X His tag on the N-terminal, we could also use a anti-His antibody to localize this protein at the subcellular level.

Initial characterization of VimF chimeric strain FLL479 showed no significant differences in growth characteristics and Rgp activity. However there was correlation

between the increase of Kgp activity to the increased hemagglutination observed. We have used W83 as positive control, although the ideal control should be the FimA mutant, as this is the site where the extra copy of *vimF* was inserted. However, even if the FimA mutant had been used, we would only expect to see a hemagglutination activity that would be lesser than W83. Contrarily we observed an increase in hemagglutination activity.

Conclusion and Future Directions

We initially raised the anti-rVimF antibody aiming to use it for localization of VimF protein in the subcellular fractions of *P. gingivalis*, thereby being able to understand the compartment where glycosylation may be taking place within the bacterial cell. However, polyclonal rabbit anti-rVimF antibody was not able to immunoreact with native VimF. Contrarily under less stringent conditions it seem to cross react with hemagglutinin domains of gingipains. Also in-silico protein sequence alignment study between VimF with RgpA, Kgp and HagA domains did not show any significant similarity. Taken together it is possible that this rabbit serum contained anti hemagglutinin antibodies. We therefore plan to purify this antibody against rVimF using affinity column. Secondly, we will use the FLL479, the VimF chimera, to purify VimF in the native state, raise antibodies and use them to investigate the subcellular localization of VimF. On the other hand, using our observation that the anti-rVimF antibody was able to react with deglycosylated protein fractions, we will use W83 and FLL479 subcellular fractions to localize the protein. These investigations will broaden our understanding on

the biology of VimF, and other important proteins involved in maturation/activation of gingipains, leading to use of this protein as a future therapeutic target.

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CHAPTER FOUR

FINAL DISCUSSION

Among the several important pathogenic bacteria implicated in adult periodontitis, *P. gingivalis* plays a central role as the keystone pathogen orchestrating other bacteria of the oral microbiota to produce the highly destructive periodontal disease which leads to exfoliation of teeth [1]. Gingipains, the cysteine proteases, represent one of the many important virulence factors responsible for pathogenic success of this organism [2]. *vimF* is one of the important genes of the *vim* locus involved in the post-translational modification of gingipains leading to its maturation/activation. Based on sequence similarity, VimF displays molecular relatedness to glycosyltransferases and is classified as glycosyltransferase I. Inactivation of this gene resulted in reduction of gingipain activity by more that 90%. In addition, the *vimF* mutant (FLL95) showed reduced hemagglutination and the inactive gingipains were not found attached to the cell [3]. Glycosylation as a post-translational modification of virulence factors has been shown to play a pivotal role in survival, virulence and pathogenesis of microorganism [4].

Cell-surface structures of organisms are decorated with sugars/glycans, one of the four fundamental building blocks of life besides proteins, nucleic acids and lipids [5]. Glycosylation, the process by which glycans are attached to proteins, is an important post-translational modification of living cells and glycoproteins are increasingly reported in prokaryotes [4]. In contrast to glycation, which is a non-enzymatic chemical reaction,

glycosylation is an enzyme-directed site-specific molecular process catalyzed by glycosyltransferases. Glycosylation in bacteria have been accepted only recently and both *N*-glycosylation and *O*-glycosylation has been reported in pathogenic bacteria[6,7] with either sequential transfer of glycans or en-block transfer of glycans to amino acids [7]. Further, unlike synthesis of nucleic acids and proteins, branching is a common feature in glycan synthesis and each step is mediated by a glycosyltransferase. In addition, glycosylation has been reported to be closely associated with the pathogenic potential of *P. gingivalis* [3,8-10]. However, detailed investigations of this complex process with regard to transfer of glycans in relation to donor and acceptor specificities have not been investigated in *P. gingivalis*. We have cloned *vimF,* purified and raised polyclonal antibody to this protein, evaluated its glycosyltransferase function and report its ability to transfer galactose to arginine specific gingipains (Rgp). In addition, our investigation suggests that VimF is a glycoprotein as well.

Further to playing an important role in post-translational modification of gingipains via glycosylation VimF is also involved in the modification of surface structures of *P. gingivalis*. We have shown that, in addition to reduced gingipain activity and growth characteristics, FLL476, the *vimF* mutant in ATCC 33277 background, showed reduced auto-aggregation and total inability to agglutinate sheep RBCs. However, there was an increase in biofilm-forming ability in this mutant. Such an increase in biofilm forming ability has also been previously observed in the *vimA* mutant of ATCC 33277 [11]. These observations strongly suggest the involvement of mature forms of gingipains on the cell surface in reduced auto-aggregation, lack of hemagglutination and change in biofilm-forming capacity observed among the *vim*

mutants. Additionally, defects in fimbrial and cell wall LPS synthesis have also been shown to influence attachment of gingipains to the cell surface, autoaggregation and biofilm formation [12]. These phenotypes are important for the invasive capacity of *P. gingivalis*.

Immature gingipains (pre and pro gingipains) undergo extensive processing before becoming mature gingipains [13]. Of the three types of gingipains, namely RgpA, RgpB and Kgp, both RgpA and Kgp are known to contain hemagglutinin/adhesion (HA) domains [14]. While studies have shown that HA domains mediate binding to host proteins such as fibronectin, fibrinogen and typeIV collagen which are all components of cellular matrix, the role of these domains, and their ability to function in the incompletely processed gingipains it is not clear [15]. It is therefore possible that HA domains of gingipains may also mediate auto-aggregation and hemagglutination. Further, our observation that the *vimF* mutants in both genetic backgrounds were non-black pigmented on BHI blood agar suggest its role in hemin assimilation. Such phenotypic variation were shown to be a function of the HA domains [12]. These surface modifications ultimately decrease the capacity of the *vimF* mutants to invade cells, suggesting an overall role of VimF in pathogenicity. These VimF mediated surface modifications were evident as shown in the electron micrographs of the *vimF* mutants compared to their respective wild-types (chapter 2). Fimbrial structures were missing in FLL476 (33277 background) and vesicles were not found in FLL95 (W83 background). It is noteworthy that gingipains are also necessary for processing of fimbriae [16]. Taken together, we have highlighted the role of VimF in the modification of surface structures

by documenting changes in the *vimF* mutant strain in a different genetic background of *P. gingivalis*.

We were able to clone and purify the recombinant VimF and raise polyclonal antibodies to this protein. Although anti-rVimF antibody reacted specifically to rVimF, the native VimF from *P. gingivalis* was not recognized by these antibodies unless first deglycosylated, suggesting VimF to be a glycoprotein. Post-translational modifications are observed as protein isoforms on a 2D gel electrophoresis [17]. We observed such isoforms when purified rVimF was subjected to 2D gel electrophoresis. Given that glycoslyctransferases can themselves be glycoproteins [18], and our observation that deglycosylated native VimF from *P. gingivalis* reacts with anti-rVimF antibody strongly suggests that VimF is a glycoprotein. rVimF was not glycosylated when expressed in *E. coli* because of the lack of glycosylation machinery in this bacteria. However glycoproteins could be expressed in *E. coli* when genes comprising glycosylation islands from other bacteria were engineered into them [19,20]. Therefore, to further understand the biology of native VimF, we have constructed a chimeric strain having an extra copy of the *vimF* gene in the *fimA* locus of W83. Studies using this chimeric strain to purify native VimF and raise antibody are currently underway.

We have reported galactosyltransferase activity of rVimF in spite of the glycosylation deficiency observed in rVimF. This observation suggests that the activity of rVimF is not contingent on the proper glycosylation of different domains of this protein. While some of the reported galactosyltransferases have been expressed and purified in *E. coli* without difficulty, others have been challenging [21-24]. In one study involving a galactosyltransferases from *Salmonella enterica*, it was shown that the C-terminal

domain was enough to exhibit the galactosyltransferase function [21]. We therefore think that either the glycosylation defect we have observed did not affect the function of rVimF or that the activity we observed may be the attenuated activity due to the glycosylation defect. The fact that galactosyltransferase are important in pathogenic bacteria is highlighted by several reports of galactosyltransferases from these bacteria [21-25].

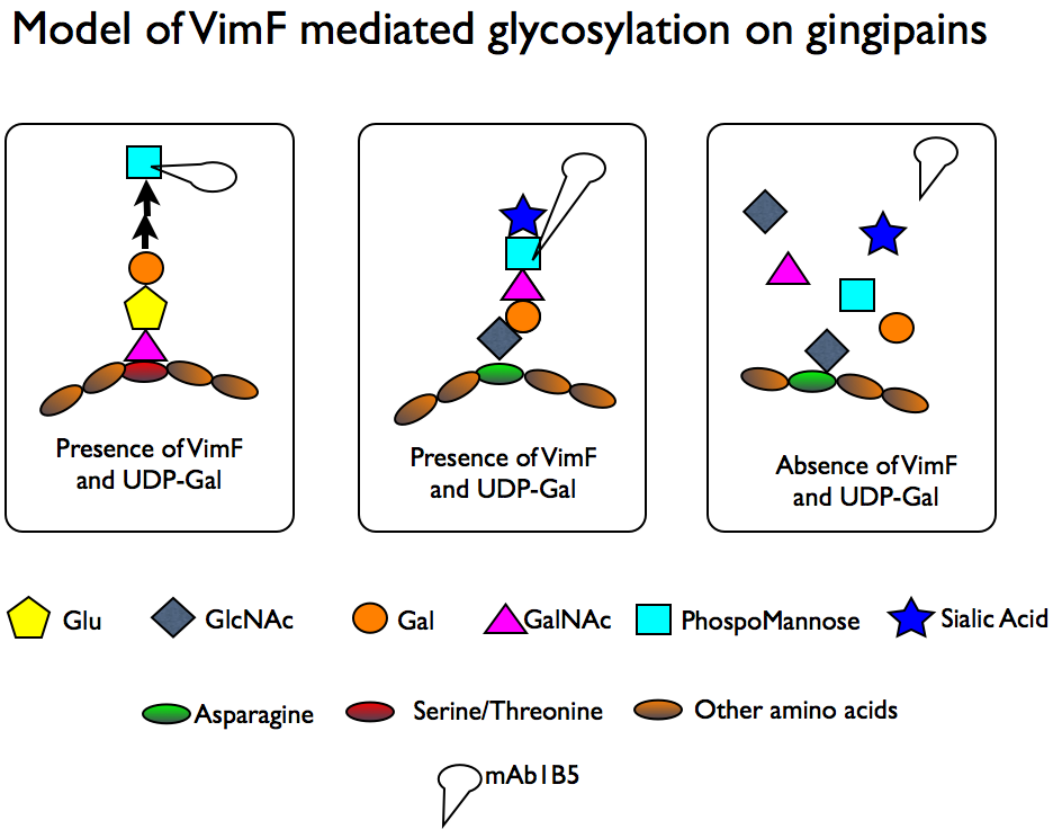

Figure 4. Model showing VimF mediated glycosylation of gingipains in *P. gingivalis*

We have demonstrated the galactosyltransferase activity of VimF by two different methods. In the first method the activity of rVimF was measured by a calorimetric assay. This assay showed the preference of rVimF to transfer UDP-galactose over UDP-glucose in the presence of *N*-acetylglucosamine. In the second method, a phosphorylated mannose specific antibody reacted with a 60 kDa band in the extracellular fraction of FLL95 only after incubation with rVimF and UDP-galactose. This observation highlights the important role galactose plays in the growing glycan chain (Fig. 4). Since glycan addition can be either sequential or en bloc, we are not sure what the case may be for the rVimF mediated transfer. Further studies need to be done to understand the prerequisite of such a VimF mediated reaction. However, the VimF mediated glycosylation via galactose transfer seem to be crucial as without this step subsequent addition of glycans, including phosphorylated mannose does not appear to be possible. This crucial step mediated by VimF makes it an interesting target for therapy. Although it has been previously reported that galactose is a major glycan present on RgpA [26] the exact position that galactose occupy in the glycan chain is not known. Finally, it is clear from our studies that the appropriate glycosylation may be a prerequisite for proteolytic processing of gingipains and the addition of galactose may occur early in the process. Linkage and sequence analysis of glycans attached to gingipains will shed more light on specific linkage(s) and position of galactose. Such studies are currently underway in our lab.

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